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Differential Methylation Pattern of *ID4, SFRP1,* and *SHP1* between Acute Myeloid Leukemia and Chronic Myeloid Leukemia

To gain insight into the differential mechanism of gene promoter hypermethylation in acute and chronic leukemia, we identified the methylation status on one part of 5′CpG rich region of 8 genes, *DAB2IP, DLC-1, H-cadherin, ID4, Integrin α4, RUNX3, SFRP1,* and *SHP1* in bone marrows from acute myeloid leukemia (AML) and chronic myeloid leukemia (CML) patients. Also, we compared the methylation status of genes in AML and CML using methylation-specific PCR (MSP). The frequencies of DNA methylation of *ID4, SFRP1,* and *SHP1* were higher in AML patients compared to those in CML patients. In contrast, no statistical difference between AML and CML was detected for other genes such as *DLC-1, DAB2IP, H-cadherin, Integrin α4,* and *RUNX3*. Taken together, these results suggest that these methylation-controlled genes may have different roles in AML and CML, and thus, may act as a biological marker of AML.

Key Words : *Leukemia, Myeloid, Acute; Chronic, BCR-ABL Positive; DNA Methylation*

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INTRODUCTION

Genomic DNA hypomethylation has been observed in the peripheral blood mononuclear cells of leukemia patients and in tumor cells of patients with B-cell lymphoproliferative diseases (1). DNA methylation may affect karyotypic stability, may influence euchromatin-heterochromatin interactions, and has been correlated with disease progression (2). On the other hand, promoters of some tumor suppressive genes are often hypermethylated (3-6). For example, some patients with lymphomas do not express tumor suppressor genes because the promoters of these genes are methylated.

Leukemia is a hematological cancer of the bone marrow and lymphatic system. In leukemia, bone marrow produces a large number of abnormal white blood cells, which overwhelm the other types of blood cells, including red blood cells and platelets, thus impairing the production of normal white blood cells. In clinical classification, leukemia can be classified as acute myeloid leukemia (AML) and chronic myeloid leukemia (CML). AML is a serious and lethal disease that affects adults. CML is a clonal disease of stem cell origin that is characterized by the presence of the Philadelphia chromosome (Ph+), which has been named t(9,22)(q34:q11). Its fusion gene product, Bcr-Abl, is a constitutively active tyrosine kinase. These facts suggest that somewhat different mechanisms may be involved in AML and CML. Nevertheless, the differential factors through which they produce a different type of leukemia are not yet completely understood.

In hematopoietic malignancies, hypermethylation of several genes including *E-cadherin, DAP kinase*, estrogen receptor (*ER*) alpha, and $p15^{INK4B}$ are associated with gene inactivation (7-10). Genes, such as *DAB2IP, DLC-1, H-cadherin, ID4, Integrin α4, RUNX3, SFRP1,* and *SHP1*, has been identified as being implicated in aberrant DNA methylation during development of human malignancy (11-18). In order to gain insight into the differential epigenetic alterations in leukemia, we investigated the methylation statuses at selected locus of these genes in AML and CML patients using a methylation-specific polymerase chain reaction (MSP).

MATERIALS AND METHODS

Sample collection

Diagnostic bone marrow samples were obtained from 23

patients with AML and 21 patients with CML. The samples were gathered by the Division of Hematology/Oncology (Department of Internal Medicine, Korea University Medical Center, Seoul, Republic of Korea) and analyzed by pathologist. Institutional review board approval and informed consent were obtained (KUMC-IRB-2006011-P-1, KUMC-IRB-2006012-P-2). Of the AML patients, 17 males and 6 females were included, with ages ranging from 26 to 78 yr, at a median age of 45.61 (SD, 15.56) yr. Of the CML patients, 11 males and 10 females were included, with ages ranging from 18 to 75 yr (mean ± SD, 49.91 ± 16.53). As controls, 22 normal peripheral bloods were obtained from healthy volunteers (11 males and 10 females) ranging from 20 to 78 yr of age (mean ± SD, 45.36 ± 20.64).

Sodium bisulfite treatment and MSP

Chemical modification was performed as described previously, with minor modifications (19). In brief, 1 μg of genomic DNA was denatured by incubation with 0.2 M NaOH for 10 min at 37℃, followed by the addition of 550 μL of 3 M sodium bisulfite (pH 5.0) (Sigma, St. Louis, MO, U.S.A.) and 10 mM hydroquinone (Sigma), which was brought to a final volume of 600 μL. The mixtures were incubated at 55℃ for 16 hr, and the modified DNA was then desalted with the Wizard Clean-Up system (Promega Corp., Madison, WI, U.S.A.). We performed polymerase chain reaction (PCR) using specific PCR primers capable of distinguishing between methylated and unmethylated DNA sequences. The primers for the unmethylated and methylated DNA sequences, PCR product size, and annealing temperature are shown in Table 1. The MSP primer sets were selected at the 5′-CpG island regions of genes using the MethPrimer software (www.urogene.org). The PCR conditions were as follows: initial denaturation and hot start at 95℃ for 5 min, and cycles consisting of 30 sec at 95℃, 30 sec at the annealing temperature, and 30 sec at

Table 1. Sequences of primers and PCR conditions

Primers		5′→3′	Annealing temp. (℃)	PCR cycles	Product size (bp)	Reference/ Gene bank accession no.
DAB2IP	UF	GGT GTT GTT AAG GTA ATA GT	52	37	181	NT 008470
	UR	CCT TAC TAA ACA ACC ACA CA				
	MF	GGC GTC GTT AAG GTA ATA GC	64	32	176	
	MR	CTA AAC GAC CGC GCG ACC GA				
DLC-1	UF	AAA CCC AAC AAA AAA ACC CAA CTA ACA	55	37	172	(12)
	UR	TTT TTT AAA GAT TGA AAT GAG GGA GTG				
	MF	CCC AAC GAA AAA ACC CGA CTA ACG	55	37	172	
	MR	TTT AAA GAT CGA AAC GAG GGA GCG				
H-Cadherin	UF	GTG GGG TTT GTT TTT TGT GAG GT	56	38	260	NT 010498
	UR	CCA ACT AAA AAC ACC CAA CAA CA				
	MF	GCG GGG TTC GTT TTT CGC GAG	64	38	260	
	MR	CCG ACT AAA AAC GCC CGA CGA				
ID4	UF	GAG GTA AAG GGA GTG GAG TTG GTT	62	35	155	NT 007592
	UR	CAA CCC AAC AAC ACC TTA CAA CCC				
	MF	GAG GTA AAG GGA GCG GAG TCG GTC	65	32	155	
	MR	CAA CCC GAC GAC GCC TTA CGA CCC				
Integrin α4	UF	GTT TAG AGT TGT TTT GTG TTT TGT G	59	39	193	(15)
	UR	AAA ACT TCA AAT ACT CAC ACT ACT				
	MF	TAG AGT TGT TTC GCG TTT TGC G	59	37	186	
	MR	CTT CGA ATA CTC GCG CTA CTT				
RUNX3	UF	GTG GGT GGT TGT TGG GTT AGT GAG	59	37	130	NT 004610
	UR	CAC CTC CTC AAC CAC CAC TAC CAC				
	MF	GTC GTC GGG TTA GCG AGG TTT CGT	59	37	130	
	MR	CGA CCG ACG CGA ACG CCT CCT C				
SHP1	UF	GGG TTT GGT TGT AGG AGT TTT GTG TA	62	35	171	(17)
	UR	CTC AAC CTA CAA TCA AAA ACA ACA CAA				
	MF	TGT AGT TTT CGG AGT TAG TGT CGC GC	68	35	126	
	MR	CCT ACG ATC GAA AAC GAC GCG AAC G				
SFRP1	UF	GTG TTT GGT ATT CAG TAG GAT TTA TTT G	59	37	193	NT 009759
	UR	CAA ACC CAA ACA ATC CCA CAA C				
	MF	GCG TTG GGT ATT TAG TAG GAT TTA TTC G	59	37	130	
	MR	CGA ACC CAA CAG ATC CCA CGA				

PCR, polymerase chain reaction; temp., temperature; UF, unmethylated forward-sequence; UR, unmethylated reverse-sequence; MF, methylated forward-sequence; MR, methylated reverse-sequence.

72℃. In addition, SCK, a human cholangiocarcinoma cell line, was used as positive control for Integrin α4 gene (20).

Statistical analysis

MSP results were analyzed as a dichotomous variable based on the presence or absence of gene methylation. The MSP results of tumor and normal samples were compared and analyzed with the Pearson chi-square test (Version 12; SPSS Inc., Chicago, IL, U.S.A.). Statistical significance was defined as a *P* value of <0.05.

RESULTS

The multiple genes were found to be methylated in bone marrow from patients with AML or CML. Specifically, the frequencies of promoter hypermethylation at selected locus in the 23 AML samples were: 78.3% (18/23) for *SHP1*, 65.2% (15/23) for *ID4* and *SFRP1*, 26.1% (6/23) for *H-cadherin,* 8.7% (2/23) for *DLC-1*, and 4.3% (1/23) for *DAB2IP* and *RUNX3*. The frequencies of DNA hypermethylation at selected locus in the 21 CML samples were: 28.6% (6/21) for *SHP1*, 19.0% (4/21) for *H-Cadherin*, 14.3% (3/21) for *ID4*, 9.5% for (2/21)

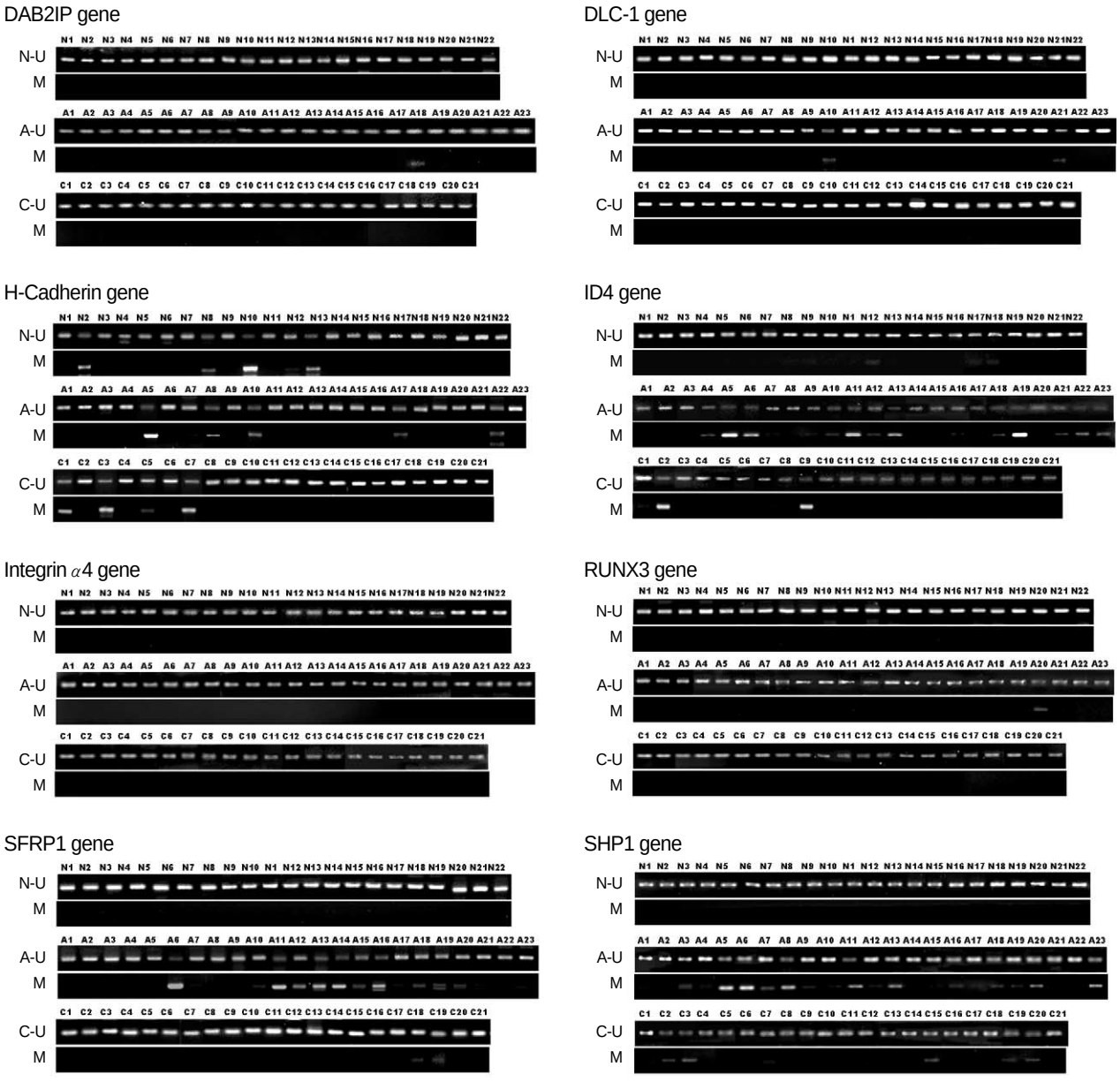

Fig. 1. PCR product images of the *DAB2IP, DLC-1, H-Cadherin, ID4, Integrin α4, RUNX3, SFRP1*, and *SHP1* genes in normal peripheral bloods, acute myeloid leukemias, and chronic myeloid leukemias.
N, normal peripheral blood; A, acute myeloid leukemia; C, chronic myeloid leukemias; U, amplified products used as primers for the unmethylated sequence in normal peripheral blood; M, amplified products used as primers for the methylated sequence.

Table 2. Methylation frequencies in normal peripheral bloods, acute myeloid leukemias, and chromic myeloid leukemias

	DAB2IP	*DLC-1*	*H-cadherin*	*ID4*	*Integrin alpha 4*	*Runx3*	*SFRP1*	*SHP1*
N	0/22 (0%)	0/22 (0%)	5/22 (22.7%)	3/22 (13.6%)	0/22 (0%)	0/22 (0%)	0/22 (0%)	0/22 (0%)
A	1/23 (4.3%)	2/23 (8.7%)	6/23 (26.1%)	15/23 (65.2%)*	0/23 (0%)	1/23 (4.3%)	15/23 (65.2%)*	18/23 (78.3%)*
C	0/21 (0%)	0/21 (0%)	4/21 (19.0%)	3/21 (14.3%)	0/21 (0%)	0/21 (0%)	2/21 (9.5%)	6/21 (28.6%)

*$P<0.05$.

N, normal peripheral bloods; A, acute myeloid leukemia; C, chronic myeloid leukemia.

for *SFRP1*, and 0% (0/21) for *DAB2IP, DLC-1, Integrin α4*, and *RUNX3* (Fig. 1). However, promoter hypermethylation of the 22 normal peripheral bloods was observed less frequently (Table 2).

There was a statistically significant difference between normal peripheral bloods and AML with respect to the frequencies of methylation of *ID4, SFRP1,* and *SHP1* (Pearson chi-square test; $P<0.0001$, $P<0.0001$, and $P<0.0001$, respectively) and between normal peripheral bloods and CML with respect to the frequencies of *SHP1* methylation (Pearson chi-square test; $P=0.007$). Furthermore, there was a statistically significant difference between the DNA methylation frequencies of AML patients and CML patients. The frequencies of DNA methylation of *ID4*, *SFRP1,* and *SHP1* were higher in AML compared to those in CML ($P=0.001$, $P<0.0001$, and $P=0.001$, respectively) (Table 2). In contrast, no statistical differences between AML and CML were detected in other genes such as *DLC-1, DAB2IP, H-cadherin, Integrin α4*, and *RUNX3*. Promoter methylation results at selected locus of eight genes with MSP method were shown in Fig. 1. These results suggest that there may be differential epigenetic modification between AML and CML.

DISCUSSION

Differential epigenetic alteration is observed in AML and CML patients. The results of the present study demonstrate the substantially increased frequency of promoter hypermethylation in *ID4, SFRP1,* and *SHP1* genes in AML compared to CML.

Leukemia, a heterogeneous group of hematopoietic malignancies that occur worldwide, includes acute and chronic myeloid leukemia. Despite many important advances in understanding the different biological and cytogenetic aspects of acute and chronic leukemia, a number of questions remained unanswered. It is understood that some kinds of leukemia present specific cytogenetic alterations (21). Different types of leukemia usually have specific epigenetic modifications that cause the activation of oncogenes and, in particular, the formation of abnormal fusion genes such as *AML1-ETO* (22, 23), a fusion protein resulting from t(8,21) translocation that commonly occurs in AML. Another typical example is the fact that Bcr-Abl is a constitutively active, cytoplasmic tyrosine kinase that is generated by t(9;22) translocation in more than 95% of CML (24).

The central role of epigenetic modification of these genes in leukemia development promoted them as reasonable targets for understanding the differential mechanism between acute and chronic leukemia. In the present study, *ID4, SFRP1,* and *SHIP1* were hypermethylated in AML compared to CML. These results suggest that the epigenetic modification of these genes may play specific roles in the development of AML rather than CML and, thus, promoted them as ideal targets for drug development to treat AML. In the future, it is clearly imperative to understand the patho-physiological differential mechanisms of these genes in AML, and to finally explore novel therapeutic strategies.

In present study, methylation frequencies of *SHP1* gene were the high detected in CML as well as AML. *SHP1* is a member of the SHP family of proteins, cytoplasmic protein tyrosine phosphatase (PTP), and has been known as a candidate tumor suppressor gene in lymphoma, leukemia and other cancers (25). Also, the hypermethylation of *SHP1* gene was frequently detected in several human cancers and the reduced expression of the *SHP1* gene in various types of leukemias and lymphomas mainly occurred by promoter methylation (26-28). Therefore, these results indicate that aberrant DNA methylation of *SHP1* gene may be related to the tumorigenicity of myeloid leukemia.

The methylation frequencies of the *DAB2IP, DLC-1,* and *RUNX3* genes were detected at different levels in acute and chronic leukemia patients; however, the difference was negligible. In some genes like *Integrin α4* gene, the promoter methylation was not observed in most of the cases. However, hypermethylation of these genes was frequent event in esophageal squamous cell carcinomas, lung cancers, and gastric cancers (11, 12, 15, 16). Thus, DNA methylation of these genes may be not related to tumorigenicity of AML/CML in contrast with other cancers.

In summary, we have identified that aberrant DNA methylation of *SHP1* is a frequent event in AML and CML. Also, the frequencies of DNA methylation of several methylation-controlled genes, including *ID4, SFRP1*, and *SHP1*, were higher in AML patients compared to those in CML patients. Although these results should be confirmed, more comprehensive studies are necessary involving various known risk factors such as smoking and occupational carcinogens, and genetic susceptibilities. Our results suggest that aberrant DNA methylation of *SHP1* may be related to the tumorigenicity

of AML and CML and hypermethylation of *ID4, SFRP1*, and *SHP1* genes may contribute to the pathogenesis mechanism of AML specifically.

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