Identification of novel genomic regions with aberrant cytosine methylation in hematological malignancies

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Abstract

To identify novel hematologic tumor-specific differentially methylated regions, we performed DNA methylation analysis of 7 new candidate genes (*ZARI*, *GATA4*, *CDH22*, *SOX3*, *SLC16A5*, *EHD3* and *TBPL1*), which has been identified to be aberrantly methylated in human cancers or animal models of human cancer. Bone marrow or peripheral blood samples from 20 patients with leukemia (including 10 AML and 6 ALL) before treatment and normal lymphocyte cells from 4 healthy individuals as well as eight human leukemia/lymphoma cell lines were used in this study and analyzed with quantitative DNA methylation analysis using the Sequenom MassARRAY system. All four normal control samples showed no or very low level of cytosine methylation in all of the genomic regions examined. However, leukemia/lymphoma cell lines showed frequent hypermethylation of the *ZARI*, *SLC16A5*, *EDH3*, *GATA4*, *CDH22* and *SOX3* genes. DNA hypermethylation of *SOX3* was also frequently observed in most of hematologic malignancy cases. Aberrant hypermethylation of *ZARI*, *GATA4* and *CDH22* was also frequently observed in both Philadelphia chromosome positive and negative ALL samples but not in all 10 AML cases. We identified novel genomic regions of *ZARI*, *GATA4*, *CDH22* and *SOX3* with aberrant cytosine methylation in hematological malignancies. Among those, aberrant DNA methylation of *ZARI*, *GATA4* and *CDH22* may be involved in carcinogenesis of lymphoid populations.

Key Words: Aberrant hypermethylation, hematological malignancies, ALL, AML

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Introduction

DNA cytosine methylation in CpG dinucleotides is an important epigenetic mechanism and directly involved in the modulation of transcriptional activity and other genome functions. The methylation in CpG-rich promoter regions generally results in transcriptional repression, whereas demethylation plays important roles in transcriptional activation through interaction of transcription factors and/or chromatin-associated protein families. Frequent mutations affecting epigenetic regulators of histone methyltransferases including EZH2 and MLL2, histone demethylases such as UTX and JMJD2C and histone acetyltransferases including CBP and p300 were identified in hematologic malignancies¹⁾. Not only

genetic mutation but also epigenetic perturbations including aberrant DNA methylation have been described during carcinogenesis of hematopoietic cells²⁾. Thus, both mutations of epigenetic pathway and perturbation of epigenomic state may play important roles in hematologic malignancies.

Aberrant methylation of specific genes, such as *BCL6*, *CEBPA*, *PRC2* genes, tumor suppressors of *CDKN2B*, *HOXD8*, *MLF1* and *PCDH8* has been described in hematological malignancy^{3, 4)}. The development of the technique for genome-wide DNA methylation analysis has allowed us to find perturbed epigenetic patterns including many aberrantly methylated regions in leukemia/lymphoma, and the reports indicated that some of these aberrantly DNA methylated regions could provide independent predictive value of companion biomarkers for therapeutics, prognostics and survival^{5, 6)}. Those genome-wide surveys of aberrantly methylated genes in leukemia may, however, miss some candidate genes, because of technical pitfalls of the genome wide surveys.

In this report, in order to identify new aberrantly

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Table 1. Patients' characteristics

Case	Age	Sex	Diagnosis	FAB classification	Karyotype	Leukocyte count at diagnosis (/mm ³)
1	56	F	AML	M2	46, XX, t(8;21)(q22;q22), t(6;7)(p23;q34?) [3/3]	21,000
2	14	F	AML	M2	46, XX, add(7)(q36), del(8)(q12?), add(11)(p15), add(21)(q22) [4/4]	61,000
3	54	M	AML	M2	46, XY [20/20]	4,300
4	59	M	AML	M2	46, XY [16/16]	400
5	35	M	AML	M2	47, XY, t(8;21)(q22;q22), del(11)(p13), +15 [10/10]	59,300
6	42	F	AML	M2	46, XX, t(8;21)(q22;q22) [17/20] 46, XX [3/20]	16,800
7	35	M	AML	M2	46, XY, t(7;11)(p15;p15) [18/18]	25,300
8	45	F	AML	M3	46, XX, t(15;17)(q22;q11) [11/11]	1,300
9	56	M	AML	M3	mitotic figure not obtained *	2,700
10	48	F	AML	M4Eo	46, XX, inv (16)(p13q22) [8/11] 46, XX [3/11]	28,700
11		M	ALL Ph -	L1	46, XY [4/4]	95,200
12	52	F	ALL Ph -	L1	46, XY, add(4)(q21), add(11)(p11?) [3/8] 46, XX [5/8]	88,400
13	43	F	ALL Ph -	L1	mitotic figure not obtained	2,900
14	40	F	ALL Ph -	L2	46, XX, t(14;19)(q32;q13?) [7/7]	17,100
15	19	M	ALL Ph +	L1	46, XY, t(9;22;14;11)(q34;q11;q32;q13), del(17)(p11.2) [16/20] 45, XY, t(9;22;14;11)(q34;q11;q32;q13), del(17)(p11.2), -20, add(21)(q12) [4/20]	265,400
16	61	F	ALL Ph +	L2	47, XX, -13, add(17)(p13?), del(22)(q11), +2mar [1/1]	14,000
17	21	M	CML CP	-	46, XY, t(3;7)(p21;q36), t(9;22)(q34;q11) [9/10] 46, XY [1/10]	9,300
18	24	F	CML CP	-	46, XX, t(9;22)(q34;q11) [5/5]	82,500
19	62	M	CML BC	lymphoid crisis	46, XY, t(9;22)(q34;q11) [17/20] 46, XY [3/20]	169,500
20	67	M	T-CLL	-	46, XY [8/8]	19,700

FAB; French America and British, AML; acute myelogenous leukemia, ALL; acute lymphoblastic leukemia, CML; chronic myelogenous leukemia, Ph; Philadelphia chromosomal translocation, CP; chronic phase, BC; blast crisis

* A PML/RARA fusion signal in 46% of the cells by fluorescence in situ hybridization

methyated regions in human leukemia/lymphoma, which may have yet to be identified, and to find surrogate end-point biomarkers for leukemia/lymphoma, we initially selected 7 candidate genomic regions, which were found to be aberrantly methylated regions in the other type of human cancers and animal models of human cancers and have never been reported to be aberrantly methylated in human leukemia. Then we examined DNA samples from hematologic malignancies by a quantitative methylation analysis using base-specific cleavage and mass spectrometry.

Materials and methods

Sample preparation of human hematologic malignancies

Bone marrow samples from 20 patients with leukemia before treatment were obtained from the Laboratory of Hematology and Rheumatology, Nihon University Itabashi Hospital (Tokyo, Japan). Clinical information of each patient is described in Table 1. Samples were

supplemental table 1.

case	disease name	position	blast (%)	comment
1	AML M2	BM	15.2	
2	AML M2	BM	50.8	
3	AML M2	BM	50.0	
4	AML M2	BM	76.2	
5	AML M2	BM	51.8	
6	AML M2	BM	38.8	
7	AML M2t(7;15)	BM	34.8	
8	APL	BM	20.6	
9	AML	BM	87.8	
10	AML M4Eo	BM	67.3	
11	ALL L1	BM	94.0	
12	ALL L1	BM	89.9	
13	ALL L1	BM	84.3	
14	ALL L2	BM	94.2	
15	ALL Ph+	BM	95.7	
16	ALL L2 Ph+	BM	99.1	
17	CML (CP)	BM	0.0	
18	CML (CP)	BM	0.0	
19	CML (BC1)	BM	96.5	
20	CLL	PB	88.0	WBC 30,100

obtained from bone marrow aspiration or peripheral blood and lymphocyte was separated from plasma of the patients using Lympho Separation Medium. Percent of blastic cells was indicated in supplemental table 1. Normal lymphocyte cells from four healthy individuals were used as normal controls. A written informed consent was obtained from all patients involved in the study. The study was approved by the Institutional Review Board of Nihon University Itabashi Hospital and Nihon University School of Medicine.

DNA was extracted from cell lines and human tumor specimens by the standard phenol/chloroform purification method or with QIAamp DNAMini Kit (Qiagen, Valencia, CA). 1 µg DNAs were extracted from each samples and applied for the methylation analysis.

Selection of candidate aberrant methylated regions

We have preliminary surveyed tissue specific and developmental specific differentially methylated regions (DMRs) in the mouse genome⁷⁾ as well as tissue specific DMRs in the human⁸⁾. In addition, previous studies have been searched for aberrant methylation in mouse models of human tumor^{9, 10)}, the other animal models¹¹⁾ and the other types of human tumors¹²⁻¹⁵⁾. The search allows us to find candidate aberrantly methylated regions, where differential methylation was identified both in normal tissue development and in some kind of tumors. Among those regions, 58 loci were identified as differentially methylated region in animal models¹⁶⁾. Among them, we selected 7 candidate genes (Table 2), whose CpG island structure were conserved between human and mouse and aberrant methylations have not been identified in human hematopoietic malignancies.

Quantitative analysis of DNA methylation

Bisulfite modification was performed by the sodium bisulfite method with an EZ DNA Methylation-Gold Kit (Zymo Research). The bisulfite-treated genomic DNA was amplified by HotStarTaq DNA Polymerase (Qiagen) (15 min at 94°C followed by 45 cycles of 20 s at 94°C, 30 s at 56°C, and 1 min at 72°C with a 3-min final extension at 72°C) with specific PCR primers (Operon Biotechnology).

A Sequenom MassARRAY quantitative methylation analysis using the MassARRAY Compact System (Sequenom) was performed for the quantitative DNA methylation analysis. This system is based on MS for the detection and quantitative analysis of DNA methylation using homogeneous Mass Cleave (hMC), base-specific cleavage and MALDI-TOF MS¹⁷⁾. Briefly, genomic DNA (1 mg) was converted with sodium bisulfite using the EZ DNA methylation-Gold kit (Zymo Research). The primers were designed using Methprimer (<http://www.urogene.org/methprimer/index1.html>) or Methy Primer Express1 Software v1.0 (Applied Biosystems).

The primer pairs were designed to span the conserved human genomic region as indicated. Bisulfite-treated DNA (up to 20 ng/ml) was amplified using HotStarTaq DNA Polymerase (Qiagen) in a 5 ml reaction volume using PCR primers at a 200 nM final concentration (See Table 2 for primer information). The PCR products were checked by agarose gel electrophoresis. After Shrimp Alkaline Phosphatase treatment, 2 ml of the PCR products were used as a template for *in vitro* transcription and RNase A Cleavage for the T-reverse reaction, as per the manufacturer's instructions (Sequenom hMC). The samples were desalted and spotted on a 384-pad SpectroCHIP (Sequenom) using a MassARRAY nanodispenser, followed by spectral acquisition on a MassARRAY Analyzer Compact MALDITOF MS (Sequenom). The resultant methylation calls were performed by EpiTYPER software v1.0 (Sequenom) to generate quantitative results for each CpG site or an aggregate of multiple CpG sites. Twice M.Sss-I-treated DNA was prepared according to the manufacture's protocols (New England Biolabs) at 37°C for 1 h followed by 65°C for 20 min inactivation for 100% methylation samples. The non-applicable reading and its corresponding site were eliminated in calculation.

Results

All four normal leukocytes showed less than 25% methylation level within all examined regions, suggesting demethylation of normal leukocyte DNAs at the candidate genes, while 6 out of 7 candidate regions showed frequent hypermethylation in 8 examined lymphoma/leukemia cell lines (Table 3 and Supplemental Figure 1a). By the analysis of DNA methylation status of the 7 genes in 20 samples from hematopoietic malignancy patients, hyper-methylation of *SOX3* was prominent both in AML and ALL. Six out of 10 AML, all 6 ALL samples showed 50% or higher methylation level at *SOX3* locus. Hyper-methylation of *ZAR1*, *GATA4* and *CDH22* were observed in ALL samples. All of 6 ALL showed 50% or higher methylation level at *ZAR1* locus. Five at *GATA4* and 3 at *CDH22* were also hyper-methylated. No AML samples reached 50% or higher methylation level at *ZAR1*, *GATA4* and *CDH22*. Aberrant methylation was not observed at *SLC16A5*, *EHD3* and *TBPL1* in any patients' samples examined.

Discussion

This study presents new genes showing aberrant hyper-methylation in the human leukemia genome. A large number of aberrantly methylated regions in hematopoietic malignancies have been reported within or close to the gene. At the present time, DNA hypomethylating agents, such as 5-azacitidine and 5-aza-2'-deoxycytidine,

Table 2.

Gene	Localization (GRCh37/hg19)	Primer Name	Sequence (5'-3')
ZAR1	chr4:48,492,968-48,493,341	ZAR1-F	TTAAGGTTTAGAGGAGGGGGAGGT
		ZAR1-R	ACAAAACCAAACCCAAAAAACAC
GATA4	chr8:11,566,658-11,566,891	GATA4-F	GTTTTTTTTGAGATGGTGTAGGGT
		GATA4-R	ATCCAAACCATTATAATCAATTCCTACA
CDH22	chr20:44,879,951-44,880,228	CDH22-F	TTGGGGTTTAGGAGTATGGA
		CDH22-R	TCCAAATACTACCTAATAATCC
SOX3	chrX:139,586,021-139,586,308	SOX3-F	GGTGGTAGGTATATGTTGATTA
		SOX3-R	CCTACAATACAACCCAATAATA
SLC16A5	chr17:73,084,269-73,084,519	SLC16A5-F	GGATTAGAAAAGTTTTTTTGTGTTT
		SLC16A5-R	AAACAAACAATAAAACCTCATACC
EHD3	chr2:31,457,288-31,457,571	EHD3-F	GGTTGGGTTTGGTTTATAGGAGTAGG
		EHD3-R	AAATTTCTTAAACCCCTCACTCACC
TBPL1	chr6:134,274,165-134,274,456	TBPL1-F	TTGATTGGGGATTAGGAAG
		TBPL1-R	CTCCCAATCTCCATAAAACT
		Forward-tag	aggaagagag
		Reverse-tag	cagtaatagactactataggagaaggct

Primers used for MassARRAY EpiTYPER

Primers used for PCR amplification of the bisulfite-converted DNA. Each reverse primer has a T7-promotor tag for in vitro transcription (5'-cagtaatagactacta tagggagaaggct-3') and the forward primer is tagged with a 10mer to balance Tm (5'-aggaa-gagag-3').

Table 3. Summary of methylation status of candidate genes at each homologous genomic region analyzed by the EpiTYPER

		<i>ZAR1</i>	<i>GATA4</i>	<i>CDH22</i>	<i>SOX3</i>	<i>SLC16A5</i>	<i>EHD3</i>	<i>TBPL1</i>
Location of the region	Chromosome	4	8	20	X	17	2	6
	Physical map (bp)	48,492,968-48,493,341	11,566,658-11,566,891	44,879,951-44,880,228	139,586,021-139,586,308	73,084,269-73,084,519	31,457,288-31,457,571	134,274,165-134,274,456
human samples from leukemia patients								
AML	Case 1	29.2%	36.3%	23.6%	53.2%	5.3%	18.9%	7.7%
	Case 2	23.0%	18.1%	19.4%	50.1%	10.8%	16.7%	13.7%
	Case 3	37.5%	26.1%	18.8%	24.0%	17.1%	16.9%	7.9%
	Case 4	31.3%	29.7%	16.3%	18.0%	12.2%	26.5%	7.9%
	Case 5	28.7%	27.0%	34.8%	51.2%	8.3%	15.2%	8.7%
	Case 6	30.4%	41.3%	35.6%	65.6%	6.7%	14.1%	7.3%
	Case 7	42.1%	49.0%	24.9%	45.3%	32.3%	31.2%	?
	Case 8	44.8%	30.4%	15.0%	61.1%	12.2%	24.8%	8.2%
	Case 9	48.1%	20.3%	17.7%	26.7%	7.5%	18.3%	6.9%
	Case 10	35.3%	28.1%	18.5%	64.2%	13.0%	24.1%	9.7%
Ph-ALL	Case 11	81.9%	68.9%	63.7%	79.3%	8.4%	16.4%	7.4%
	Case 12	81.4%	65.7%	58.1%	86.1%	25.5%	23.8%	7.7%
	Case 13	50.8%	96.0%	37.3%	88.2%	23.5%	19.6%	?
	Case 14	70.8%	71.7%	45.8%	72.6%	14.1%	16.5%	4.4%
Ph+ALL	Case 15	67.1%	43.6%	44.0%	78.1%	27.3%	18.4%	5.4%
	Case 16	71.1%	78.0%	62.0%	77.5%	15.7%	19.3%	7.8%
CML CP	Case 17	39.5%	10.7%	21.9%	21.0%	19.5%	25.3%	32.4%
	Case 18	20.1%	25.1%	13.3%	49.0%	7.7%	20.0%	11.6%
CML BC	Case 19	79.6%	65.3%	29.3%	56.9%	11.8%	23.7%	?
T-CLL	Case 20	49.1%	44.9%	31.8%	44.1%	48.5%	55.2%	7.8%
Normal lymphocytes from healthy volunteers								
	HV1	24.3%	19.0%	14.0%	20.8%	17.7%	27.8%	6.2%
	HV2	22.9%	15.9%	16.8%	24.0%	20.4%	22.7%	5.5%
	HV3	23.1%	15.1%	13.0%	16.9%	15.3%	23.6%	11.8%
	HV4	25.3%	17.9%	23.9%	20.8%	8.8%	23.7%	6.4%

The average methylation levels of the entire target regions are shown. Genes methylated more than 50% are indicated by gray color. Genome location of each region is shown according to UCSC genome browser, Feb2009 GRCh37/hg19.

Supplemental Figure 1.

DNA methylation level at specific loci of leukemia samples analyzed by Sequenom MassARRAY. The epigram shows the percent DNA methylation level of each CpG site of the target region. Methylation level is shown as a color heatmaps. The yellow circle indicates 100% methylation, and the red circle is 0% methylation at each CpG site. The number of CpG sites, target sequence length and sample names are shown in each epigram. Supplemental Figure 1a shows human leukemia/lymphoma cell line results from 8 lymphoma/leukemia cell line DNA samples and 4 healthy volunteer lymphocyte samples as control. Supplemental Figure 1b shows results of 20 human sporadic hematologic malignancies from patients and 4 normal lymphocyte controls from healthy volunteers.

b

have been used for patients with myelodysplastic syndrome (MDS). The use of these drugs results in DNA hypomethylation and gene re-expression, both *in vivo* and *in vitro*. Because hypermethylation of the promoters of certain tumor suppressor genes is prevalent in MDS and AML, it is postulated that the DNA hypomethylation induced by DNA demethylating agents may result in the reactivation of silenced genes, restoring their cancer suppressing functions, and inducing cellular differentiation¹⁸). However, most studies have failed to show a relationship between induction of DNA hypomethylation and clinical response. Those facts indicate that there still exist critical unknown genes epigenetically modified in hematological malignancies.

This is the first report to show aberrant methylation of the *SOX3* gene in leukemia/lymphoma samples. *SOX3* belongs to SOX gene family, encoding transcription factors that act as key regulators of tissue differentiation in embryogenesis. SOXB1 subgroup genes including *SOX1*, 2 and 3 are widely expressed in neural cells, involved in neural development and differentiation¹⁹). Dysfunction of *SOX3* protein results in failure of cognitive and pituitary development, leading to mental retardation and growth hormone deficiency in human. Little is known about the involvement of *SOX3* in tumorigenesis. Presence of antibodies for *SOX3* and the other member of SOXB1 family proteins in the serum of small-cell lung cancer (SCLC) patients have been reported, but the role of *SOX3* in SCLC development is still unclear²⁰). It is interesting that the hyper-methylation of *SOX3* was observed both in AML and ALL samples, even though two malignant cells develop after the differentiation from hematopoietic stem cell. The result indicates that the aberrant methylation appears in early hematopoietic lineages before those two distinct commitments. In addition this aberrant methylation could be common event to induce malignancy in both AML and ALL.

In the present study, different DNA methylation patterns were observed in *ZAR1*, *GATA4*, and *CDH22* between AML and ALL samples. Preferential aberrant hypermethylation was observed in ALL rather than AML, although aberrant DNA methylation is a dominant mechanism in MDS progression to AML and abundant in MDS and secondary AML^{21, 22}). Ji H et al. showed that myelopoiesis and lymphopoiesis result in different terminal DNA methylation patterns and, that myeloid commitment is characterized by less global DNA methylation than lymphoid commitment²³). Gaudet F, et al. demonstrated not only that constitutive DNA methylation was required in the establishment and maintenance of the hematopoietic stem cell (HSC) self-renewal program, but they showed also hypomethylated HSCs intrinsically lack lymphoid developmental potential, whereas they maintain normal myeloid and megakaryoerythroid potential²⁴). Although the mechanism of stem cell differ-

entiation and development of leukemic cell (or probable leukemic stem cell) could be different, some DNA methylation pattern might be associated with leukemiagenesis and its differentiation to myeloid or lymphoid populations. Aberrant DNA methylation of *ZAR1*, *GATA4*, and *CDH22* may be associated with differentiation to lymphoid populations of leukemic stem cell.

ZAR1 and *GATA4* are known to be involved in fetal development. For instance, *ZAR1* is engaged in oocyte-embryo transition, and *GATA4* is expressed in yolk sac and associated with heart formation. However, little is known about its role in hematological malignancies. Hyper-methylation of the *ZAR1* genes in human melanomas and brain tumors were reported recently. Shinojima et al. noted a very high frequency of hypermethylation of the *ZAR1* intergenic region in human melanomas, in contrast to non-methylation in both normal human epidermal melanocyte cell lines and melanocytic nevus surgical specimens⁹). Watanabe et al. reported that hypermethylation of *ZAR1* in various types of human brain tumors²⁵).

Seven candidate aberrantly methylated genomic regions, which have yet to be identified by whole genome scan, were analyzed in this study. In four out of seven candidate genes aberrantly methylated in leukemia have been identified. This high frequent detection could be because we screened candidate genes based on the evidence obtained from previous studies in both human and animal model of human cancers. Even though there are some limitations in the study using animal models, there are several advantages, as they have a uniform genetic background, could be kept in a controlled environment, and prone to develop tumors of the same etiology. Those merits let us find out new human candidate genes, which have not been found by the whole genome scan analysis and may be a surrogate marker for clinical endpoints. Since our present data clearly showed that the candidate loci found in the analysis of mouse tumor samples were also aberrantly methylated in human leukemia samples, we are planning to explore more genomic region in DNA methylation and gene expression based on the analysis of mouse tumor samples. Further comparative genomic analysis may elucidate a critical unknown gene epigenetically modified in hematological malignancies.

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