

Dense methylation of types 1 and 2 regulatory regions of the CD10 gene promoter in infant acute lymphoblastic leukemia with MLL/AF4 fusion gene

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Abstract: Infant ALL displays distinct biologic and clinical features with a poor prognosis. The CD10-negative immunophenotype of infant ALL is a hallmark and provides a predictable signature of MLL rearrangement. While CD10 negativity reflects an earlier stage of B-cell development, complete IgH gene rearrangements (VDJH), found in almost half of the patients, show more mature IgH status. Discordance between immunophenotype and genotype of infant ALL suggests an aberrant process in immunophenotypic steps of differentiation or a secondary down-regulation of CD10 expression. In this study, CD10-negative infant ALL with MLL/AF4, CD10-positive infant ALL with germ-line MLL, CD10-positive pre-B ALL cell line, infant AML (M5) with MLL/AF9 and pediatric AML (M2) with AML1/ETO were analyzed for VDJH status and methylation of CD10 gene promoters. Three of the four infant ALL samples showed complete rearrangements of the VDJH gene with productive joints. Bisulfite sequencing of CD10 type 1 and 2 promoters showed that more than 84% of the CpG dinucleotides identified were methylated in all three CD10-negative infant ALL samples with MLL/AF4. The CpG dinucleotides distributed in the clusters of putative Sp1-binding sites and functionally active regulatory regions of the promoters were fully methylated. In contrast, none of the CpG dinucleotides were methylated in the CD10-positive ALL samples. Structural evidence of dense methylation in the CD10 gene promoter suggested that methylated transcription factor binding sites contributing to CD10 silencing as an epigenetic mechanism.

TABLE I. Clinical Features, CD10 expression and MLL Rearrangements of the Leukemic Samples

Characteristics	iALL-1	iALL-2	iALL-3	iALL-4	Pre-B CL	iAML	pAML
Age	0 m	15 m (R)	11 m	7 m	17 y	8 m	5 y
Sex	female	female	male	male	female	male	female
WBC (/ μ L)	112,000	2,200	54,700	38,920		11,100	7,120
Karyotypes	t(4;11)	t(4;11)	t(4;11)	normal	hypodiploid	t(9;11)	t(8;21)
Fusion genes	MLL/AF4	MLL/AF4	MLL/AF4	-	-	MLL/AF9	AML1/ETO
sCD10 (%)	<1	1.3	0.2	42.8	99.4	<1	<1

iALL, infant ALL; CL, cell line; iAML, infant AML; pAML, pediatric AML; sCD10, surface CD10; m, months; y, years; R, relapse; WBC, white blood cell count at diagnosis

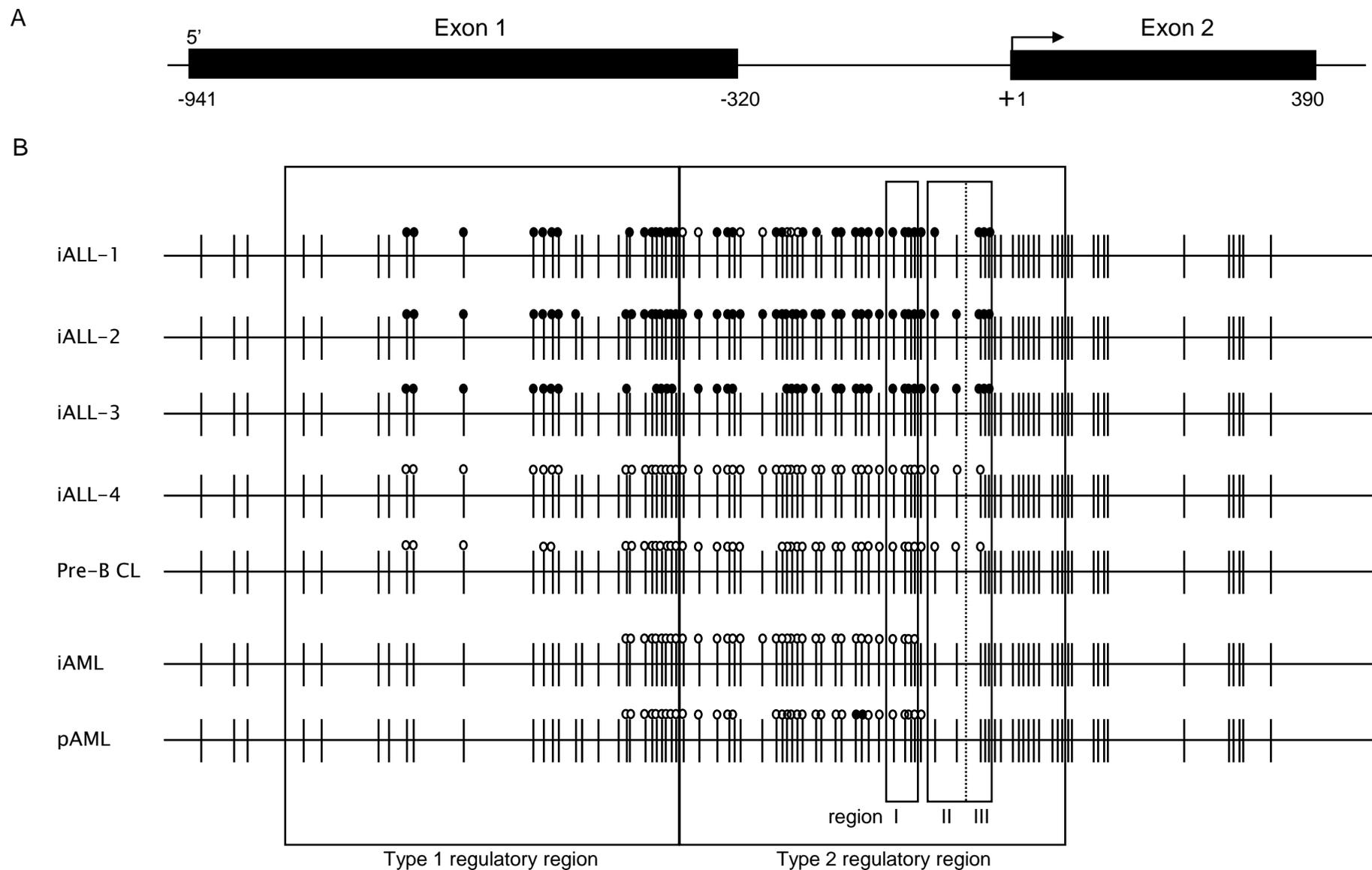
TABLE II. VDJ_H Rearrangement Status of Infant ALL

	VDJ _H status	
	Allele 1	Allele 2
iALL-1	V _H 6-1/D _H 6-19/J _H 4 (out of frame/nonproductive)	V _H 2-26/D _H 3-3/J _H 6 (in frame/productive)
iALL-2	V _H 1-02/D _H 3-22/J _H 4 (out of frame/nonproductive)	V _H 2-26/D _H 3-3/J _H 6 (in frame/productive)
iALL-3	ND	ND
iALL-4	V _H 5/D _H nd/J _H nd (out of frame/nonproductive)	V _H 1-03/D _H 1-26/J _H 5 (in frame/productive)

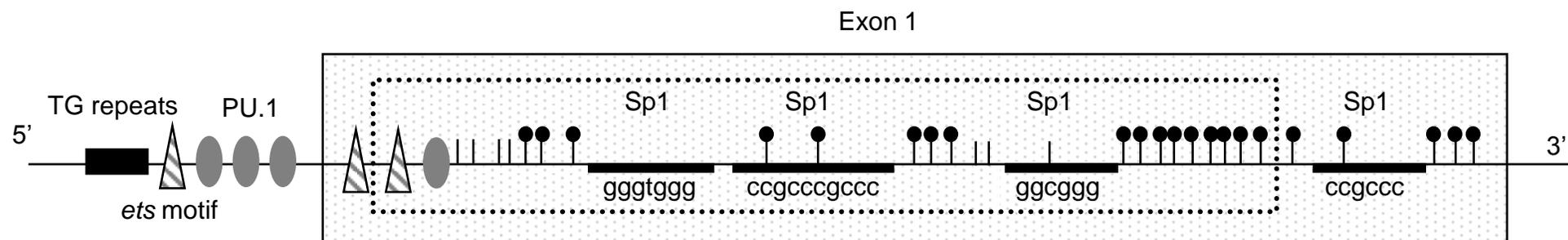
iALL, infant ALL; ND, not detected; nd, not defined

TABLE III. Number of CpGs and Methylation Status of ALL and AML

Number of CpGs	iALL-1	iALL-2	iALL-3	iALL-4	Pre-B CL	iAML	pAML
Type 1 and 2 regulatory region (66)							
Analyzed	44	48	36	45	41	34	33
Unmethylated	7	0	0	45	41	34	31
Methylated	37	48	36	0	0	0	2
methylated/ analyzed (%)	84	100	100	0	0	0	6.1
Type 1 regulatory region (24)							
Analyzed	15	17	12	16	14	9	9
Unmethylated	0	0	0	16	14	9	9
Methylated	15	17	12	0	0	0	0
methylated/ analyzed (%)	100	100	100	0	0	0	0
Type 2 regulatory region (42)							
Analyzed	29	31	24	29	27	25	24
Unmethylated	7	0	0	29	27	25	22
Methylated	22	31	24	0	0	0	2
methylated/ analyzed (%)	76	100	100	0	0	0	8.3



A



B

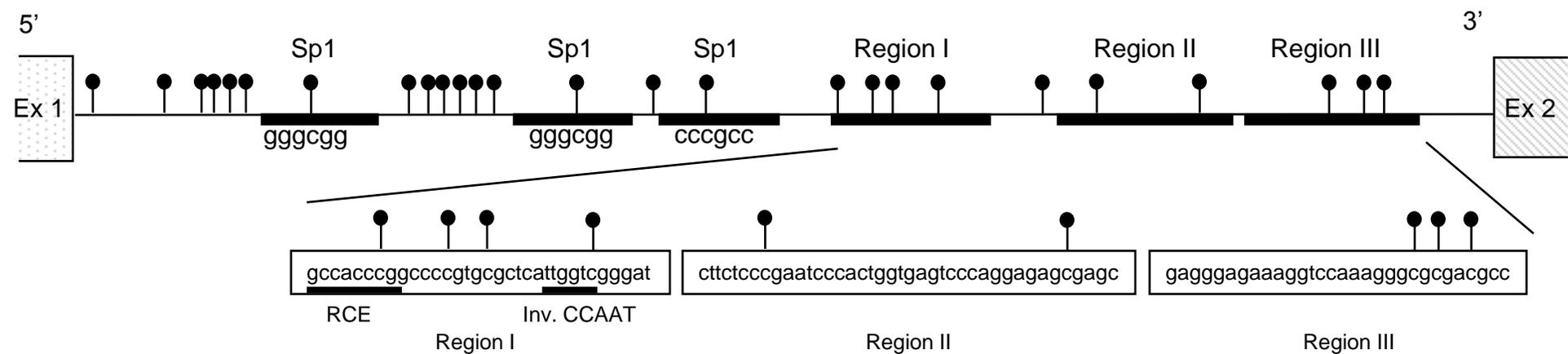
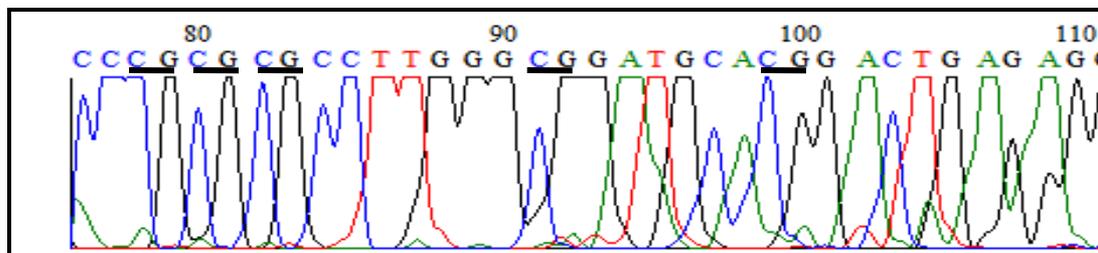


Fig. 3

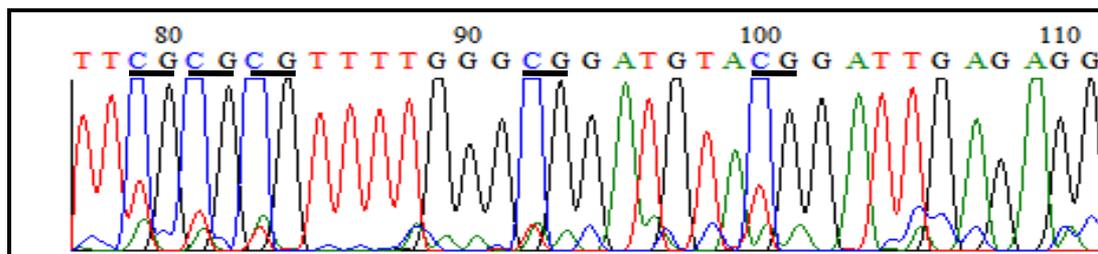
A

CCCGCGCGCCTTGGGCGGATGCACGGACTGAGAGG

B



C



D

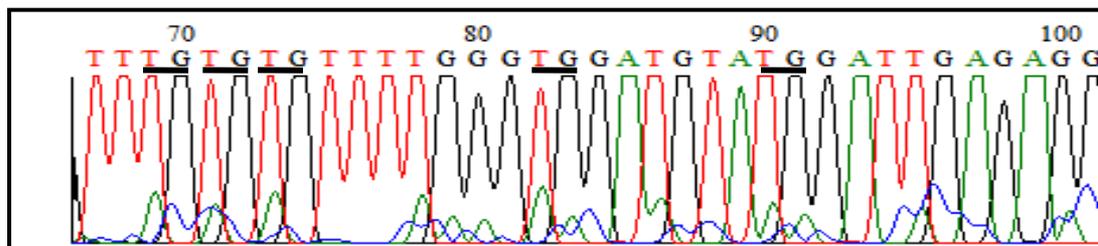


Fig. 1

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**Dense Methylation of Type 1 and Type 2 Regulatory Regions of the CD10 Gene Promoter
in Infant Acute Lymphoblastic Leukemia with MLL/AF4 Fusion Gene**

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ABSTRACT

Infant ALL displays distinct biologic and clinical features with a poor prognosis. The CD10-negative immunophenotype of infant ALL is a hallmark and provides a predictable signature of MLL rearrangement. While CD10 negativity reflects an earlier stage of B-cell development, complete IgH gene rearrangements (VDJ_H), found in almost half of the patients, show more mature IgH status. Discordance between immunophenotype and genotype of infant ALL suggests an aberrant process in immunophenotypic steps of differentiation or a secondary down-regulation of CD10 expression. In this study, CD10-negative infant ALL with MLL/AF4, CD10-positive infant ALL with germ-line MLL, CD10-positive pre-B ALL cell line, infant AML (M5) with MLL/AF9 and pediatric AML (M2) with AML1/ETO were analyzed for VDJ_H status and methylation of CD10 gene promoters. Three of the four infant ALL samples showed complete rearrangements of the VDJ_H gene with productive joints. Bisulfite sequencing of CD10 type 1 and 2 promoters showed that more than 84% of the CpG dinucleotides identified were methylated in all three CD10-negative infant ALL samples with MLL/AF4. The CpG dinucleotides distributed in the clusters of putative Sp1-binding sites and functionally active regulatory regions of the promoters were fully methylated. In contrast, none of the CpG

dinucleotides were methylated in the CD10-positive ALL samples. Structural evidence of dense methylation in the CD10 gene promoter suggested that methylated transcription factor binding sites contributing to CD10 silencing as an epigenetic mechanism.

INTRODUCTION

Infant acute leukemia occurs within the first year of life and displays distinct biologic and clinical features with a poor prognosis.¹⁻⁴ Seventy to 90% of patients harbor rearrangements of the mixed-lineage leukemia (MLL) gene at the chromosomal region 11q23.^{5,6} The acute lymphoblastic leukemia (ALL) subtype of infant acute leukemia predominantly includes the t(4;11)(q21;q23) region with the MLL/AF4 fusion gene, followed by the t(11;19)(q23;p13) region with the MLL/ENL fusion gene and the t(9;11)(p22;q23) region with the MLL/AF9 fusion gene.^{7,8} Recent gene expression profiling studies indicate that MLL-rearranged (MLL-R) ALLs are arrested at an early lymphoid progenitor stage of development.^{9,10} Among these highly distinct expression patterns in MLL-R, a total lack of CD10 expression is a hallmark and provides a predictable signature of MLL rearrangements.^{9,11}

CD10, the neutral endopeptidase 24.11, is a regulator of B-cell growth and proliferation.¹² The enzyme degrades a number of bioactive peptides with various functions that depend on the cell type or tissue of origin. CD10 is expressed in a biphasic pattern that correlates with specific stages of B cell development. CD10 is detected on precursor B (pro- and pre-B) cells and lymph node germinal center B cells but is not detected on lymphoid progenitor cells, early pro-B cells,

mature B cells (naïve B-cells and mantle cells) or plasma cells.¹³ This biphasic pattern is generally maintained in malignant counterparts.¹³

Immunoglobulin (Ig) gene rearrangements are regarded as a molecular fingerprint of B cell development.¹⁴ CD10 is believed to be expressed during the first stages of Ig heavy-chain (IgH) rearrangement and can be co-expressed with surface μ -VpreB or μ - λ 5 pre-BCR.¹⁵ Several lines of evidence indicate a high frequency of complete IgH rearrangements (VDJ_H) in CD10-negative pro-B ALL.¹⁶ Ig gene rearrangement patterns were related to the presence and type of MLL rearrangement but not to the age at diagnosis or to ALL-CD10 expression.^{16,17} Discordance between the genotype and the immunophenotype of infant ALL with respect to cellular maturity suggested an aberrant process in the steps of immunophenotypic differentiation or a secondary down-regulation of CD10 expression associated with MLL rearrangements.

Methylation of promoter-associated cytosine-guanine (CpG) islands is an epigenetic modification of DNA and has been associated with gene silencing and malignant transformation.^{18,19} An inverse correlation was previously observed between methylation of the CD10 promoter and CD10 expression in adult²⁰ and pediatric ALL²¹ as well as in human prostate cancer.²² However, data on the frequency of methylation in the various types of ALL, particularly in

infant ALL with MLL-R, remain scarce. We therefore performed methylation analysis of the entire promoter region of the CD10 gene in infant ALL to investigate epigenetic mechanisms responsible for CD10 negativity. We describe dense methylation of the CD10 promoter with particular emphasis on the methylation of transcription factor binding sites within the CD10 promoter (type 1 and 2 regulatory regions) in CD10-negative MLL/AF4 infant ALL carrying a productive rearrangement of the VDJ_H allele.

MATERIALS AND METHODS

Cell Samples

Peripheral blood (PB) or bone marrow (BM) samples from MLL/AF4 infant leukemia (3 samples), acute myeloid leukemia (AML) (2 samples, AML1/ETO and MLL/AF9, respectively) and a sample of infant pre-B ALL (MLL-germline, MLL-G) were obtained after informed parental consent and with approval of the Ethics Committee of the Kanazawa University Hospital. The pre-B ALL cell line (HAL-01) was from RIKEN Bank (Tsukuba, Ibaraki, Japan). Mononuclear cells were isolated from PB or BM samples by Ficoll-Paque density gradient

centrifugation. DNA was extracted and purified according to the manufacturer's instructions using the QIAamp DNA Blood Mini Kit (Qiagen, Hilden, Germany).

The immunophenotype of leukemic cells was assessed by flow cytometry using a dual-laser FACS Calibur (Becton Dickinson, San Diego, CA, USA). The resulting data were analyzed using the CELL Quest software (Becton Dickinson). Immunophenotypic subclassification was done according to the guidelines of the European Group for the Immunological Characterization of Leukemias (EGIL): Pro-B ALL, CD19+/CD10-/cytoplasmic (cy) IgM-/surface (s) IgM-; common ALL, CD19+/CD10+/cy IgM-/s IgM- and Pre-B ALL, CD19+/CD10+/cy IgM+/s IgM-. Criteria for immunophenotypic marker positivity were: expression on $\geq 20\%$, or $\geq 10\%$ of blasts for cell surface and cytoplasmic markers, respectively.

Detection of Rearranged Leukemia-associated Genes

The presence of t(4;11)(q21;q23) with the MLL/AF4 fusion gene, t(9;11)(p22;q23) with the MLL/AF9 fusion gene and t(8;21)(q22;q22) with the AML1/ETO fusion gene was demonstrated by one or more of the following methods: metaphase cytogenetic analysis, polymerase chain reaction (PCR) with reverse transcription (RT-PCR) and fluorescence *in situ* hybridization

(FISH) as part of the diagnostic work-up.

Polymerase Chain Reaction (PCR) and Sequence Analysis of IgH Gene Rearrangements

PCR amplification of IgH-VDJ rearrangements was performed in a Thermocycler (GeneAmp PCR system 9700, Applied Biosystems) using a series of seven V_H family FR1 consensus primers and a J_H consensus primer by modification of a method previously described.

²³ Clonal PCR products were purified using QIAquick gel extraction kits (QIAGEN, Hilden, Germany), directly sequenced using the BigDye-Terminator v3.1 cycle sequencing kit and were analyzed on a 3100 sequencer (Applied Biosystems). V_H, D_H, J_H segments and their family members were identified with the closest matching known human germ line genes using the IGBlast search (<http://www.ncbi.nlm.nih.gov/igblast/>, National Center for Biotechnology Information, Bethesda, MD). The criteria used for D_H gene determination were: a minimal homology of six matches in a row or seven matches interrupted by one mismatch.

Bisulfite Modification of DNA, PCR Amplification, and Sequence Analyses of the CD10

Promoter Regions

All methylation studies were done by bisulfite modification of DNA which converts all unmethylated cytosine-phosphate-guanosine (CpG) sites to uracil-phosphate-guanosine (UpG), leaving methylated CpGs intact. Bisulfite treatment of genomic DNA was done using MethylEasy Xceed (Human Genetic Signatures, North Ryde, Australia). The DNA was then amplified with the following gene-specific primers: Type 1-1, sense 5'- TTTATAGGGGAGAGATGGAG-3'; antisense 5'- ACCAAAAAAAAAAAAACAATTCC- 3'; Type 1-2, sense 5'- TTTTGGTTTTGTAGTGGT-3'; antisense 5'- CAATCAAACCTTCACCACTTA-3'; Type 2-1, sense 5'- TTYGGTTTTAGTTTGGAGTT-3'; antisense 5'- CCCTTTAAACCTTTCTCCCT-3'; Type 2-2, sense 5'- TGTGGGTGTGGGTTGGAGGGATG-3'; antisense 5'- CCCTCCCCATGCCCATCCCAC-3'.

Following sequencing of the amplified DNA, methylated CpGs were identified by visual inspection of the sequencing traces in the electropherograms and by comparison with sequencing traces of the CD10 gene sequence relative to the reference GenBank sequence (accession number X79438).

RESULTS

Clinical Features, Immunophenotype and MLL Rearrangements of the Leukemic Samples

The clinical features of seven samples and the characteristics of their leukemic cells are described in Table I. Four of the five samples (<12 months of age) of infant leukemia (iALL-1 to -4, and iAML) and a pediatric AML (pAML) sample (5 years of age) were studied at diagnosis. A case with congenital ALL was analyzed at day 1 of age for initial diagnosis (iALL-1) and at 15 months of age for relapse (iALL-2). A cell line (Pre-B CL) established from a 17-year-old female with pre-B ALL was also studied. Three of the four infant ALL samples (iALL-1 to -3) were t(4;11)-positive ALL with the MLL/AF4 fusion gene and one sample (iALL-4) showed a normal karyotype. The iAML and pAML samples were t(9;11)-positive with the MLL/AF9 fusion gene and t(8;21)-positive with the AML1/ETO fusion gene, respectively. CD10 expression, determined by the flow cytometric immunophenotyping, was negative ($\leq 20\%$) in all three MLL/AF4-positive ALLs (iALL-1 to -3) with a pro-B ALL immunophenotype (CD19+/CD34+/cyIgM-/sIgM-). In contrast, MLL-G ALLs (iALL-4 and Pre-B CL) expressed CD10 and the common ALL immunophenotype (CD19+/CD34-/cyIgM-/sIgM-).

IgH Gene Rearrangements (VDJ_H) in Infant ALL

Sequence analyses of PCR-amplified VDJ_H segments are summarized in Table II. In two CD10-negative infant ALL (iALL-1 and -2) and a CD10-positive infant ALL (iALL-4), two major clonal VDJ_H rearrangements were identified, in which each sequence contained either an out-of-frame joining (a nonproductive rearrangement) or an in-frame joining without a stop codon (a productive rearrangement). In iALL-3, clonal PCR products were not detected following repeated amplification, indicating that the IgH genes remained germ line or that there was only DJ joining. These recombination processes were consistent with a hierarchical order of IgH gene rearrangements in normal B precursor cells.¹⁴ In addition, in the case with congenital leukemia, biclonality of the leukemic cells was shown by a difference in the usage of V_H and D_H gene family members in nonproductive rearrangements determined at diagnosis (iALL-1) and relapse (iALL-2). These results indicated that CD10 negativity was not correlated with the developmental status of IgH gene rearrangements in infant ALL.

Methylation of the CD10 Promoter in Infant ALL

Figure 1 contains electropherograms of base sequences, from nt -211 to nt -177, for two representative samples (i-ALL-2 and pre-B CL) with bisulfite treatment. Cytosine residues other than CpG dinucleotides were all converted to thymine, whereas methylated cytosine residues in CpG dinucleotides were retained as cytosine, indicating that bisulfite treatment was completed. The CD10 gene promoter contains two separate regulatory regions that control the transcription of 5' alternatively spliced CD10 transcripts.²⁴ The type 1 regulatory region contains the exon 1 sequence within nt -821 to nt -326 relative to the major transcription initiation site at the beginning of exon 2. The type 2 regulatory region spans the sequence from nt -326 to nt +105 (Fig. 2).^{25,26} In addition, three functionally active transcription factor binding sites, designated as regions I to III, have been identified in the type 2 regulatory region (Fig. 2B).²⁵ A total of 66 CpG dinucleotides are present within these two regulatory regions (24 in the type 1 regulatory region and 42 in the type 2 regulatory region) (Fig. 2 and Table III). Mapping of the CpG dinucleotides showed that they were densely distributed within the 3'-half portion of the type 1 regulatory region and over the entire type 2 regulatory region (Fig. 2B). At least 33 of these 66 CpG dinucleotides were successfully analyzed by bisulfite sequencing (Table III, Fig. 2B). We quantified CpG methylation as the methylated CpG content (%) in a single gene by showing the

number of CpG methylation present in the CD10 promoter region. This analysis showed that more than 84% of the identified CpG dinucleotides in all three CD10-negative infant ALL with MLL/AF4 were methylated. Comparison of the two regulatory regions revealed no difference in methylation frequency. In contrast, none of the CpG dinucleotides were methylated in the CD10-positive ALL (iALL-4 and Pre-B CL) and only 2 of the CpG dinucleotides were methylated in pAML samples (Table III, Fig. 2B).

Figure 3 illustrates the CD10 promoter methylation status and transcription factor binding sites in the representative iALL-2 sample.²⁷ The 5' UTR exon 1 sequence contained multiple putative PU.1 binding sites, consensus *ets*-binding motifs and a cluster of four potential Sp1-binding sites (Fig. 3A). The CpG dinucleotides within the Sp1-binding sites, as well as in the surrounding sites were highly methylated. In the type 2 regulatory region (Fig 3B), 31 of the 42 CpG dinucleotides were analyzed and all 31 were methylated. Notably, three putative Sp1-binding sites, as well as functionally active transcription factor binding sites in regions I to III, contained 12 (39%) of these methylated CpG dinucleotides. These results indicated that both type 1 and 2 regulatory regions in the CD 10 gene promoter were highly methylated in CD10-negative infant ALL with MLL/AF4 and provided structural evidence of dense

methylation of the transcription factor binding sites.

DISCUSSION

We have demonstrated dense methylation patterns in both type 1 and 2 regulatory regions of the CD10 promoter in CD10-negative MLL/AF4 infant ALL bearing a productive, rearranged VDJ_H allele. In addition, we are the first to describe the precise methylated sites within the CD10 gene promoter.

Two separate regulatory regions have been identified in the CD10 gene promoter.²⁵⁻²⁷ The type 1 and 2 regulatory regions share common features characterized by the presence of multiple transcription initiation sites and the absence of classic TATA boxes and consensus initiator elements. The purine-rich type 1 regulatory region includes the 5' UTR exon 1 sequence with multiple putative PU.1 binding sites and consensus *ets*-binding motifs.^{27,28} The distribution map of CpG dinucleotides showed a concentration of methylated CpG dinucleotides within the 3'-half portion of the type 1 regulatory region (Fig 2A), in which a cluster of four potential Sp1-binding sites is located (Fig 3A). Clustering of Sp1-binding sites and their cooperative activation of transcription has been reported for several genes with a TATA-less-promoter.²⁸

Therefore, Sp1-binding sites observed in exon 1 of the CD10 gene are anticipated to contribute to type 1 promoter activity. Indeed, a factor(s) immunologically related to Sp1 specifically binds to the most upstream GC-rich sequence (ggtggg) and is required for optimal promoter activity.

²⁶ Bisulfite sequencing revealed that the CpG dinucleotides in the putative Sp1-binding sites were almost all methylated in three samples of infant ALL with MLL/AF4. The sequence upstream of exon 1 that contains multiple putative PU.1 sites and *ets*-binding motifs also showed a positive influence on type 1 CD10 promoter activity. ²⁷ However, it is unlikely that methylation is involved in the action of these regulatory elements since there are no CpG dinucleotides in the consensus sites.

The GC-rich type 2 regulatory region identified in the intron upstream of exon 2 ²⁷ contains numerous CpG dinucleotides (Fig 2). Strikingly, 31 CpG dinucleotides were distributed in the type 2 regulatory region and all were methylated in a sample of infant ALL (iALL-2) (Fig 2). Such high frequency of methylation was consistently seen in an additional two samples of CD10-negative ALL (Fig 2). The CD 10 type 2 regulatory region contains multiple putative Sp1-binding sites, a potential consensus retinoblastoma control element (RCE) and an inverted CCAAT box. ^{25, 27} Upregulation of Sp1-mediated transcription by retinoblastoma protein

through the RCE has been reported.²⁹ Recently, three functionally active transcription factor binding sites, designated as regions I to III, have been identified in the type 2 regulatory region.²⁵ CBF/NF-Y was shown to bind the inverted CCAAT box in region I and is responsible for tissue-specific CD10 expression.²⁵ Bisulfite sequencing indicated complete methylation of the CpG dinucleotides in the three putative Sp1-binding sites and within regions I to III in infant ALL with MLL/AF4 (Fig 3B). Specific methyl-CpG dinucleotides within the promoter may inhibit *trans*-factors that preferentially bind to sequences containing CpG dinucleotides by limiting DNA access to the factors or by conformational alteration of DNA structure or they may act as a guide for methyl-CpG-binding proteins (MeCP-1 and MeCP-2) that modulate transcriptional activity.³⁰⁻³² Thus, structural evidence of dense methylation in two regulatory regions of the promoter suggests that methylated transcription factor binding sites contribute to CD10 silencing as an epigenetic mechanism. Since our study contained no MLL/ENL or MLL/AF9 ALL samples, extended analyses including MLL-R subtypes are required to specify MLL-R involvement in methylation of the CD10 promoter.

Three types of CD10 transcripts, types 1, 2A and 2B, have been identified resulting from alternative splicing of 5' untranslated regions; the type 1 regulatory region promotes type 1

transcripts from exon 1 and the type 2 regulatory region promotes types 2A and 2B transcripts initiated within exon 2 in a tissue-specific manner.²⁷ Type 2 transcripts are more abundant than type 1 transcripts in a variety of cell types, while some CD10-positive pre-B cell ALL cell lines have also been shown to contain high levels of type 1 transcripts.²⁷ Although the relative abundance of type 1 and type 2 transcripts was not examined in our clinical samples, hypermethylation of both of the regulatory regions is consistent with a total lack of CD10 expression in infant ALL with MLL/AF4.

Taylor et al.¹³ have shown that the CD10 promoter region is consistently not methylated in normal lymphocytes at early and late stages of maturation regardless of the CD10 expression level, while in B-cell malignancies methylation of the CD10 promoter is common at specific stages of maturation and is frequently associated with absence of CD10 expression. These data suggest that the biphasic pattern of CD10 expression in normal differentiation of B cells is controlled by tissue- and stage-specific regulatory mechanisms other than DNA methylation. However, CD10 methylation plays a role in lymphoid malignancies by silencing CD10 expression. Further investigations are required for complete elucidation of the role of methylation in CD10 expression because epigenetic modulation involves not only DNA

methylation but also histone modifications and chromatin remodeling that interact in an epigenetic network.^{33,34}

In conclusion, our results show structural evidence of DNA promoter methylation suggesting a contribution to CD10 silencing as an epigenetic mechanism. Functional analyses such as transfection of MLL fusion genes are required to confirm the precise involvement of MLL-R in methylation. Further investigation of epigenetic network interactions associated with MLL will provide an additional insight into the aberrant transcriptional program in MLL leukemia.

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FIGURE LEGENDS

Fig. 1. Electropherograms of base sequences with or without bisulfite treatment.

- A. Reference sequence of the CD10 promoter region, from nt -211 to nt -177 (GenBank accession number: X 79438). The CpG dinucleotides are underlined.
- B. Electropherogram of iALL-2 without bisulfite treatment.
- C. Electropherogram of iALL-2 with bisulfate treatment.
- D. Electropherogram of pre-B CL with bisulfate treatment.

Fig. 2. Methylation status of the CD10 gene promoter region in leukemic cells.

- A. Structural illustration of the 5' UTR exon 1 and exon 2 of the CD10 gene promoter region.
The arrow delineates the major transcription initiation site (+1)
- B. Distribution maps of the CpG dinucleotides and their methylation status. Closed and open circles indicate methylated and unmethylated CpG dinucleotides, respectively. The vertical bars represent the position of CpG dinucleotides.

Fig. 3. CD10 promoter methylation status and transcription binding sites in iALL-2.

A. Putative binding sites for the *ets*, PU.1 and Sp1 transcription factors within the 5' UTR

proximal to exon 1. The dashed box delineates the type 1 regulatory region. Methylated CpG dinucleotides are indicated as in Fig. 1.

B. Transcription factor binding sites within the type 2 regulatory region. RCE and inv. CCAAT

indicate a potential consensus retinoblastoma control element and an inverted CCAAT box, respectively.

To Review Officials:

Point-by-point responses to the comments of the reviewers are as follows.

1. In the abstract, at the bottom line the authors state "None or few of the CpG dinucleotides were methylated." It is unclear what they mean by few. This should be much more specific in an abstract.

A1: "A few of the methylated CpG dinucleotides" indicate 2 of the methylated CpG observed in a pAML case as shown in Figure 2. However, as indicated by the reviewer, the description in the abstract should be concisely focusing on the methylation status of ALL. Thus, we have amended the description in the abstract as follows: "In contrast, none of the CpG dinucleotides were methylated in the CD10-positive ALL samples", on page 2, line 16.

Following the amendment, we have changed the description in the results as follows: "In contrast, none of the CpG dinucleotides were methylated in the CD10-positive ALL (iALL-4 and Pre-B CL) and only 2 of the CpG dinucleotides were methylated in pAML samples (Table III, Fig. 2B)", on page 13, line 4.

2. On page 4 fourth line from the bottom, the authors state that "The enzyme is a cell surface aminopeptidase". It is not an aminopeptidase, but an endopeptidase.

A2: We have amended the description of this enzyme as follows: "The enzyme degrades a number of bioactive peptides with various functions that depend on the cell type or tissue of origin", on page 4, line 13.

3. On page 10, the authors state that CD10 was absent in all three MLL/AF4 positive ALL's. They do not indicate how this was determined and the methodology used to make this statement.

A3: We have amended the description of CD10 expression as follows: “CD10 expression, determined by the flow cytometric immunophenotyping, was negative ($\leq 20\%$) in all three MLL/AF4-positive ALLs (iALL-1 to -3) with a pro-B ALL immunophenotype (CD19+/CD34+/cyIgM-/sIgM-)”, on page 10, line 12.