Expression of myeloperoxidase in acute myeloid leukemia blasts mirrors the distinct DNA methylation pattern involving the downregulation of DNA methyltransferase *DNMT3B*

Key words; myeloperoxidase, acute myeloid leukemia, DNA methylation, DNA methyltransferase 3 beta

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Abstract

Myeloperoxidase (MPO) has been associated with both a myeloid lineage commitment and favorable prognosis in patients with acute myeloid leukemia (AML). DNA methyltransferase inhibitors (decitabine and zeburaline) induced *MPO* gene promoter demethylation and *MPO* gene transcription in AML cells with low MPO activity.

Therefore, *MPO* gene transcription was directly and indirectly regulated by DNA methylation. A DNA methylation microarray subsequently revealed a distinct methylation pattern in 33 genes, including DNA methyltransferase 3 beta (*DNMT3B*), in CD34-positive cells obtained from AML patients with a high percentage of MPO-positive blasts. Based on the inverse relationship between the methylation status of *DNMT3B* and *MPO*, we found an inverse relationship between *DNMT3B* and *MPO* transcription levels in CD34-positive AML cells (p=0.0283). In addition, a distinct methylation pattern was observed in 5 genes related to myeloid differentiation or therapeutic sensitivity in CD34-positive cells from AML patients with a high percentage of MPO-positive blasts. Taken together, the results of the present study indicate that MPO may serve as an informative marker for identifying a distinct and crucial DNA methylation profile in CD34-positive AML cells.

1 Introduction

The expression of myeloperoxidase (MPO), a microbicidal protein, which is measured by cytochemistry in leukemia blasts, is widely accepted as a golden marker for the diagnosis of acute myeloid leukemia (AML) in the French-American-British (FAB) and WHO classifications because its expression is tightly regulated in a lineage-specific

manner, and cytochemistry is also a more time- and cost-efficient method than other molecular analyses such as the expression of normal and / or abnormal genes.

The expression of MPO provides critical information regarding not only the phenotype of AML cells, but also the prognosis of AML patients [1-5]. Overall survival in AML patients with a high percentage of MPO-positive blasts defined by routine cytochemical staining (>50% is defined as high MPO enzymatic activity) was shown to be significantly better than that in patients with a low percentage of MPO-positive blasts when treated with intensive chemotherapy, which enhances the toxicity of chemotherapy leading to a good response to treatment [6, 7]. In addition to the direct therapeutic effect of MPO, we also demonstrated that the prognosis of AML patients with high expression levels of *MPO* mRNA in CD133-positive cells, which contain a putative AML stem/progenitor compartment, was significantly better than that in patients with low expression levels [8]. This finding indicated that *MPO* gene transcription in leukemia stem/progenitor cells correlated with distinct genetic and/or epigenetic alternations related to sensitivity to chemotherapeutic drugs.

Although the regulation of *MPO* gene expression has been investigated extensively [9-12], its regulation in leukemia cells remains largely unknown. Transcription factors such as RUNX1 and CEBPA, whose binding sites are located on the *MPO* gene, are

considered to be important positive regulators of *MPO* transcription in normal hematopoietic cells [11, 13, 14]. However, the leukemia blasts of t(8;21) AML, which has a RUNX1-RNXT1 fusion protein, were shown to be strongly positive for MPO [15, 16], though RUNX1-RUNXT1 acts in a dominant negative manner for RUNX1 [17]. Similarly, the very strong association between high MPO positivity of blasts and the presence of CEBPA double mutation was demonstrated in AML patients [18], despite the dominant negative function of CEBPA double mutation for wild-type CEBPA [19]. The dominant negative manner in RUNX1-RUNXT1 and CEBPA double mutation may not be involved in the regulation of *MPO* transcription in AML blasts. These contradictions suggest that the regulation of *MPO* gene transcription in leukemia cells differs from that in normal hematopoietic cells, and also that the control of *MPO* gene expression cannot be attributed solely to the function of transcription factors.

The epigenetic control of gene expression through DNA methylation has been suggested to play important roles in determining the biological behavior of cells, including leukemogenesis [20-22]. The degree of DNA methylation in mononuclear cells obtained from untreated AML patients was shown to be inversely correlated with the expression of *MPO* [23]. However, because *MPO* gene expression is known to be tightly regulated in tissue- and differentiation-dependent manners, the DNA methylation

pattern in highly purified leukemia stem / progenitor cells needs to be evaluated. Figueroa ME, et al. reported distinct DNA methylation profiles in CD34-positive leukemia cells from AML with the CEBPA double mutation, AML1-ETO, and CBFb-MYH11 fusion genes, the blasts of which show very high MPO positivity [24]. Based on these findings, we hypothesized that *MPO* gene transcription in CD34-positive AML cells was regulated by the DNA methylation machinery, and that the expression of *MPO* mirrored the specific DNA methylation profile linked to critical biological differences in CD34-positive AML cells. To prove this hypothesis, we investigated changes in DNA methylation patterns and *MPO* gene expression following the exposure of 5 AML cell lines with low MPO enzymatic activity to DNA methyltransferase inhibitors (DNMT-is), and performed a comprehensive analysis of genome-wide DNA methylation profiles in CD34-positive cells obtained from 20 patients.

2 Materials and methods

2.1 Cell staining

Cells were morphologically analyzed using cytospin slides stained by the May-Grunwald-Giemsa method, diaminobenzidine (DAB) method, and with an

anti-MPO antibody. MPO protein expression and its enzymatic activity were shown as an average of the percentages of MPO (protein or activity)-positive cells independently evaluated by two investigators. The positivity of MPO enzymatic activity by the DAB method was divided into 2 categories; high positivity (>50%) of MPO enzymatic activity (MPOa-H) and low positivity (≤50%) of MPO enzymatic activity (MPOa-L).

2.2 Culture of cell lines treated with DNA methyltransferase inhibitors

Human leukemia cell lines were purchased from the following institutions; SKM-1 and CMK-86 from the Japanese Collection of Research Bioresources (Tokyo, Japan; JCRB0118, IFO50428); KG-1, KG-1a, THP-1, and K562 from the Institute of Physical and Chemical Research (Tsukuba, Japan; RCB1166, RCB1928, RCB1189, RCB1897); CML-T1 and BV173, from Deutschen Sammlung von Mikroorganismen und Zellkulfuren (Braunschweig, Germany; ACC-7, ACC-20); Kasumi-1 and SU-DHL-6, from the American Type Culture Collection (Manassas, VA, USA; CRL-2724, CRL-2959). SKM-1 and Kasumi-1 were categorized as MPOa-H cell lines, while the other 8 cell lines were MPOa-L cell lines. A detailed description regarding the cultivation with exposure to DNMT-is (decitabine (Dac) or zebularine (Zeb)) can be found in the Supplemental Experimental Procedures.

2.3 Cell purification from clinical samples

Bone marrow samples were collected after obtaining approval from the Ethical Committees of the participating hospitals. CD34-positive cells were selected after Ficoll density gradient centrifugation using a magnet bead method (CD34 MicroBead Kit, MACS, Gladbach, Germany). The CD34-positive bone marrow cells of 2 healthy volunteers were purchased from Lonza Walkersville Inc. (Walkersville, MD, USA). The purity of CD34-positive cells assessed by flow cytometry was more than 95% after selection.

2.4 Genomic DNA and total RNA extraction, and cDNA synthesis

High molecular weight genomic DNA and total RNA were extracted using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany) and TRIzol reagent (Invitrogen, CA, USA), respectively. cDNA was synthesized using oligo-dT primers and Super Script III Reverse Transcriptase (Invitrogen).

2.5 Quantitative reverse transcriptase-polymerase chain reaction for *MPO* and *DNMT3B* genes

MPO, DNA methyltransferase 3 beta (DNMT3B), and *Abelson tyrosine-protein kinase 1 (ABL1)* transcription levels were quantitated using a quantitative reverse transcriptase-polymerase chain reaction assay (QRT-PCR) and shown as MPO/ABL1 and DNMT3B/ABL1 ratios. Detailed conditions including primer sequences are shown in the Supplemental Experimental Procedures.

2.6 DNA methylation microarray by Illumina infinium assay

This assay was performed as described previously [25]. Briefly, 4μL of bisulfite-converted DNA (150ng) was used for the whole-genome amplification reaction. After amplification, DNA was fragmented enzymatically, precipitated, and re-suspended in hybridization buffer. All subsequent steps were performed following the standard Infinium protocol (User Guide part #15019519 A). Fragmented DNA was dispensed onto Human Methylation450 (HM450) BeadChips, and hybridization was performed in a hybridization oven for 20 h, following which the array was processed through a primer extension and immunohistochemistry staining protocol to allow for the detection of a single-base extension reaction [26, 27]. BeadChips were coated and then imaged on an Illumina iScan.

The methylation level of each CpG locus was calculated in a GenomeStudio®

Methylation module as the methylation beta-value (β = intensity of the Methylated allele (M) / (intensity of the Unmethylated allele (U) + intensity of the Methylated allele (M) + 100).

2.7 Statistical methods

The results of the *in vitro* experiments are presented as the mean ± standard deviation of three independent experiments and were compared using a one-way analysis of variance and multiple comparison tests. Correlations between the percentage of MPO-positive cells and mRNA expression levels were estimated by Spearman's correlation coefficient by rank. Correlations between MPO and DNMT3B mRNA expression levels were estimated by linear regression analysis. Whole genome bisulfite sequencing data obtained from the Illumina infinium assay were categorized into 4 groups; MPOa-H AML group (a high percentage [>50%] of MPO-positive myeloblasts in bone marrow smear), MPOa-L AML group (a low percentage [≤50%] of MPO-positive myeloblasts in bone marrow smear), Philadelphia-chromosome positive acute lymphoblastic leukemia (Ph+ALL) group, and healthy donor group. Based on the average values of methylation for each probe in each group, all probe sets were evaluated using a one-way analysis of variance. Significance was considered at the level

of two-tailed 0.05 for all analyses.

3 Results

3.1 The introduction of demethylation and expression of the *MPO* gene by DNMT-is in leukemia cell lines

We assessed *MPO* gene expression and the DNA methylation status of the 5' region of the *MPO* gene in 10 leukemia cell lines. The MPO/ABL1 mRNA ratio was high in MPOa-H cell lines (MPO/ABL1 mRNA ratio > 100), and low in MPOa-L cells (MPO/ABL1 mRNA ratio < 1.0) (Table S1). To assess the relationship between the DNA methylation status and *MPO* gene expression, the 5' region of the *MPO* gene in these 10 cell lines was examined by bisulfite sequencing. The average methylation of the *MPO* promoter in MPOa-H cell lines was less than 25%, while that in all MPOa-L cell lines, except for one (K562), was above 60%, which was significant (p=0.0038, Figure S1a). We compared the amount of MPO mRNA before and after the DNMT-i treatment in MPOa-L AML cell lines to verify the role of DNMT. Demethylation of the 5' region of the *MPO* gene was achieved by Dac at 1.0 μM and Zeb at 50.0 μM, which was confirmed using bisulfite sequencing (Figure S1b). The results of QRT-PCR revealed that the Dac treatment significantly induced MPO mRNA in KG-1, KG-1a, and

THP-1, and a similar result was observed with Zeb in THP-1 and K562 (Figure 1). However, flow cytometry showed that induction of the intracellular MPO protein was minimal in KG-1, KG-1a, and THP-1 after the Dac treatment, and did not occur in THP-1 or K562 after the Zeb treatment (Figure S1c). Immunohistochemical analysis and cytochemistry failed to detect the MPO protein or its enzymatic activity, respectively, in any MPOa-L AML cell lines treated with DNMT-is.

3.2 Significant relationship between the percentage of MPO-positive blasts on bone marrow smears and the amount of the *MPO* gene in CD34-positive AML cells from patients

Table 1 summarizes the clinical characteristics of the 18 patients and 2 healthy donors who participated in this study. Leukemia blasts showed high (>50%, MPOa-H AML group) and low (≤50%, MPOa-L AML group) MPO positivity in 9 and 6 AML patients, respectively. Three patients had Philadelphia-chromosome positive acute lymphoblastic leukemia (Ph+ALL group). Gene mutation analysis revealed *FLT3 internal tandem duplications (FLT3-ITD)* in 5 AML patients (Unique patient number (UPN)-3, -12, -13, -14, and -15), an *NPM1* mutation in 3 AML patients (UPN-12, -13, and -15), *CEBPA* single mutation in one (UPN-4), and *CEBPA* double mutation in 3 AML patients

(UPN-6, -7, and -9). The MPO positivity of blasts measured by cytochemistry and the amount of MPO mRNA were shown in Table 1. Immunocytochemistry revealed a correlation between the percentage of MPO protein-positive blasts and the activity of MPO by cytochemistry in the CD34-positive cell fraction. All 3 AML patients with the *CEBPA* double mutation were categorized into the MPOa-H group. The DAB method revealed a significant relationship in the percentage of MPO enzymatic activity-positive cells between blasts on bone marrow smears and CD34-positive cells after purification in 15 AML patients, ($p<0.0001$). The percentage of blasts with MPO enzymatic activity on bone marrow smears and that of CD34-positive cells correlated with the amount of MPO mRNA in CD34-positive AML cells ($p<0.0001$. Figure 2, and $p=0.0009$, respectively). No MPO enzymatic activity was detected in blasts on bone marrow smears or CD34-positive cells from three Ph+ALL patients, and the amount of MPO mRNA in CD34-positive cells was low. Both were also low in samples from healthy donors.

3.3 DNA methylation status of the *MPO* gene correlated with the expression of both the *MPO* gene and its enzymatic activity in CD34-positive cells from clinical samples

To determine the methylation status of the *MPO* gene in more detail, 13 CpG sites were analyzed on its promoter and gene body in leukemia and control samples using the Illumina infinium assay with HM 450 BeadChips. This analysis revealed the low methylation status of CpG sites in both the promoter and gene body of the *MPO* gene in all AML samples that were categorized in the MPOa-H AML group (i.e. the enzymatic activity of the MPO protein and *MPO* gene expression levels were high). In contrast, hypermethylation was observed in CD34-positive cells from the MPOa-L AML group (i.e. both the enzymatic activity of the MPO protein and *MPO* gene expression levels were low), 3 Ph+ALL patients, and 2 healthy donors, who had low MPO mRNA expression levels. An inverse relationship was found between the DNA methylation status of the 13 CpG sites in the *MPO* gene and its enzymatic activity in CD34-positive cells (Figure 3). To validate the results obtained from the Illumina infinium assay, the methylation status of the CpG site at number 2 was confirmed using bisulfite sequencing. It revealed that the 5' region of the *MPO* promoter was hypomethylated in all samples from the MPOa-H AML group, while hypermethylation was noted in the same region in samples from the MPOa-L AML group (Figure S2). The average percentage of methylated sites in the *MPO* promoter fragment was significantly lower in samples from MPOa-H AML group than in those from the MPOa-L AML group

($p<0.0001$); the average percentages of methylated sites were 10.9% and 66.5% in the MPOa-H AML and MPOa-L AML groups, respectively. Similar results were obtained with the leukemia cell lines described above. Taken together, these results suggest that MPO mRNA expression is regulated in a DNA methylation-dependent manner in CD34-positive leukemia and normal bone marrow cells.

3.4 CD34-positive cells from MPOa-H AML patients had distinct epigenetic signatures

Based on results suggesting that the expression of MPO mRNA may be modulated by the methylation of DNA in AML stem / progenitor cells (CD34 positive cells), we speculated that the methylation patterns of some genes may have significantly positive or inverse relationships with that of the *MPO* gene, which could explain biological differences in the AML groups categorized by MPO expression (MPOa-H and MPOa-L). To examine this hypothesis, CD34-positive cells from 20 samples were subjected to the DNA methylation profiling of over 450,000 CpG sites using the Illumina infinium assay with HM 450 BeadChips (Figure 4a). From a total of 12,369 probe sets whose methylation status was successfully tested, 3,433 probe sets were excluded from further analysis because of the lack of their gene symbol. The remaining probe sets covered

2,658 and 3,721 CpG sites in the promoter region and gene body, respectively. Among them, there were 45 and 68 CpG sites in the promoter region and the gene body, respectively, which showed significantly positive or opposite methylation patterns between the MPOa-H AML group and 3 other groups (MPOa-L AML, Ph+ALL, and healthy donor groups), covering 49 genes, including the *MPO* gene. Among them, 34 genes including the *MPO* gene showed a distinct pattern in both the promoter region and their gene body. Nine genes (*MPO* and other 8 genes) clearly displayed hypomethylation at their CpG sites in the MPOa-H group, while those of 25 genes were hypermethylated relative to the 3 other groups (Figure 4b). To confirm that these methylation changes were not a reflection of global changes in DNA methylation, we compared the methylation status of *long interspersed nuclear element-1 (LINE-1)*, which is used as a marker of global methylation of DNA sequences [28], in the MPOa-H AML group and other groups (including the control). No significant difference was observed in the methylation of *LINE-1* between the MPOa-H and MPOa-L AML groups (Figure S3). Therefore, these results indicate that a distinct methylation pattern in 34 genes was observed in CD34-positive cells from the MPOa-H AML group.

3.5 Inverse relationship between *MPO* and *DNMT3B* gene expression in CD34-positive AML cells

Among the genes listed in Figure 4b, we focused on the *DNMT3B* gene, which has an opposite methylation pattern to that of the *MPO* gene, because of its role as a *de novo* methyltransferase [29]. We measured MPO and DNMT3B mRNA expression in CD34-positive cells from 15 AML samples (Table S2), and showed that the amount of MPO mRNA was inversely correlated with that of DNMT3B mRNA (Figure 5a. R^2=0.3189, p=0.0283). This relationship was also found in 10 AML patients with a normal karyotype out of these 15 AML patients (R^2=0.4853, p=0.0252) (Figure 5b). The amount of DNMT3B mRNA was inversely related to the percentage of MPO enzymatic activity in blasts on bone marrow smears (p=0.0689). The amount of DNMT3B mRNA was not correlated with the presence of any gene mutations (FLT3-ITD, CEBPA mutation, or NPM1 mutation).

4 Discussion

The MPO enzymatic activity of blasts from AML patients was significantly related to a specific DNA methylation pattern affecting *MPO* gene transcription in AML cells in the present study. We also demonstrated that the methylation pattern of the 33 genes,

determined by a DNA methylation microarray, distinguished the MPOa-H AML group from the other groups. Based on the opposite methylation patterns between the *DNMT3B* (hypermethylation) and *MPO* (hypomethylation) genes, the downregulated transcription of the *DNMT3B* gene was correlated with the upregulated transcription of the *MPO* gene in CD34-positive AML cells.

Schmelz K et al. reported that azacitidine induced *MPO* gene transcription in AML cell lines and AML blasts from patients [23]. However, Dac and Zeb, which have similar, but distinct functions from those of azacitidine in DNA demethylation [30-32], caused the limited induction of *MPO* transcription in some AML cell lines in the present study, in spite of the successful demethylation of the *MPO* gene in all the AML cell lines tested. This result suggests that demethylation of the 5' promoter region in the *MPO* gene is necessary, but not sufficient for *MPO* gene transcription. Hypomethylation of the gene body may also be important in *MPO* gene expression, as was shown in the present study and suggested in a previous report on chronic lymphocyte leukemia [33].

Since the function of DNMT3B is responsible for initiating *de novo* DNA methylation along with DNMT3A [34-36], it was important that the opposite DNA methylation and expression patterns were found between the *DNMT3B* and *MPO* genes (Figure 4b, 5a, and 5b). The *DNMT3A* gene is known as one of the most frequently mutated genes in

AML in up to 36% of cytogenetically normal AML patients [37-40]. Although the functional aspects of mutated DNMT3A in AML have yet to be elucidated [40, 41], several studies showed that the *DNMT3A* mutation was associated with a worse overall survival in patients with AML [37, 39, 42]. However, the biological and clinical implications of DNMT3B have not yet been clearly defined in AML. Although the *DNMT3B* mutation is not a typical event in AML, the overexpression of DNMT3B is commonly observed in various types of cancers in humans, including AML [43-46]. Hayette S, et al. reported that the overexpression of DNMT3B was an independent poor prognostic factor in AML patients [47]. DNA methylation and expression patterns between *DNMT3B* and *MPO* were found to be opposite in the present study, which suggests that DNMT3B could methylate and regulate *MPO* gene transcription. Hence, these findings indicate that DNMT3B may affect the phenotype of AML stem / progenitor cells. Further investigations on *DNMT3B* gene, including its alternative spliceoforms, are warranted in order to understand its role in leukemogenesis [48-51].

A previous study showed that the DNMT3A R882 mutation resulted in less methylation at 182 specific genomic loci in AML samples than in wild-type samples [37]. The downregulated *DNMT3B* gene expression may also contribute to the distinct DNA methylation signature observed in CD34-positive cells from the MPOa-H group.

Among genes listed in Figure 4b, five genes (that is, Proteinase 3 (PRTN3), protein phosphatase 1, a common activator of TP53, as a regulatory subunit of 13B (PPP1R13B), Nuclear factor I/A (NFIA), Homeobox C11 (HOXC11) and CD109) have been reported to be biologically and/or clinically relevant in leukemia. In MPOa-H AML cells, PRTN3 (a gene involved with chemoresistance in an AML cell line [52]) and PPP1R13B (a gene associated with prognosis in ALL [53]) may be up-regulated due to DNA hypomethylation. On the other hand, the three other genes (that is NFIA, HOXC11 and CD109) showed a hypermethylated pattern and may be downregulated. NFIA and HOXC11 are known to be involved in the myeloid differentiation of leukemic cells, [54,55] and CD109 is found on a subset of stem/progenitor cells. [56] These findings support the hypothesis that leukemia stem/progenitor cells that highly express MPO mRNA are more likely to be sensitive to chemotherapy and may represent the early process of myeloid commitment before apparent morphological differentiation; however, this needs to be confirmed in future studies.

Epigenetic regulator mutations have recently been shown to be both biologically and clinically relevant in AML. These regulators include tet methylcytosine dioxygenase 2 (TET2), isocitrate dehydrogenase 1 (IDH1), IDH2, additional sex combs-like 1 (ASXL1), and DNMT3A [37, 57-60]. Although these mutations may alter the status of

DNA methylation and gene expression, they were not analyzed in the present study. Therefore, our findings should be interpreted carefully, and they should be confirmed in larger studies that include mutation analysis for epigenetic regulators.

In conclusion, to the best of our best knowledge, this is the first study to demonstrate that *MPO* gene expression is a potential indicator of a distinct methylation signature, in which *DNMT3B* gene expression is involved. Additionally, we consider the expression of MPO in AML blasts to be an informative biomarker for epigenetic alternations in AML. Future studies are needed on the role of DNMT3B in AML and use of DNMT-i for refractory AML.

Conflict of interest statement

The authors declare no financial or personal conflicts of interest.

Author's contribution

H.I. and Y. Miyazaki conceived and designed the study; H.I., D.I., W.Y.F., S.S., K.A., Y.S., D.S., K.T., H.H., Y.I., J.T., H.T., S.Y., T.F., T.H., Y. Moriuchi, K.Y., S.N., and Y. Miyazaki collected and analyzed the samples and data; H.I. and Y. Miyazaki performed the statistical analysis, wrote the manuscript, and created the figures and tables; and all

authors critically reviewed the manuscript and read and approved the final version.

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References

1. Hoyle CF, Gray RG, Wheatley K, Wheatley K, Swirsky D, de Bastos M, et al. Prognostic importance of Sudan Black positivity: a study of bone marrow slides from 1386 patients with de novo acute myeloid leukemia. Br J Haematol. 1991; 79(3): 398-407.

2. Matsuo T, Cox C, Bennett JM. Prognostic significance of myeloperoxidase positivity of blast cells in acute myeloblastic leukemia without maturation (FAB: M1): an ECOG study. Hematol Pathol 1989; 3(4): 153-158.

3. Matsuo T, Kuriyama K, Miyazaki Y, Yoshida S, Tomonaga M, Emi N, et al. The percentage of myeloperoxidase-positive blast cells is a strong independent prognostic factor in acute myeloid leukemia, even in the patients with normal karyotype.

Leukemia. 2003; 17(8): 1538-1543.

4. Miyawaki S, Sakamaki H, Ohtake S, Emi N, Yagasaki F, Mitani K, et al. A randomized, postremission comparison of four courses of standard-dose consolidation therapy without maintenance therapy versus three courses of standard-dose consolidation with maintenance therapy in adults with acute myeloid leukemia. Cancer. 2005; 104(12): 2726-2734.

5. Ohtake S, Miyawaki S, Fujita H, Kiyoi H, Shinagawa K, Usui N, et al. Randomized study of induction therapy comparing standard-dose idarubicin with high-dose daunorubicin in adult patients with previously untreated acute myeloid leukemia: the JALSG AML201 Study. Blood. 2011; 117(8): 2358-2365.

6. Sawayama Y, Miyazaki Y, Ando K, Horio K, Tsutsumi C, Imanishi D, et al. Expression of myeloperoxidase enhances the chemosensitivity of leukemia cells through the generation of reactive species and the nitration of protein. Leukemia. 2008; 22(5): 956-964.

7. Nakazato T, Sagawa M, Yamato K, Xian M, Yamamoto T, Suematsu M, et al. Myeloperoxidase is a key regulator of oxidative stress mediated apoptosis in myeloid leukemic cells. Clin Cancer Res. 2007;13(18):5436-5445.

8. Taguchi J, Miyazaki Y, Tsutsumi C, Sawayama Y, Ando K, Tsushima H, et al.

Expression of the myeloperoxidase gene in AC133 positive leukemia cells relates to the prognosis of acute myeloid leukemia. Leuk Res. 2006; 30(9): 1105-1112.

9. Austin GE, Zhao W-G, Zhang W, Austin ED, Findley HW, Murtagh JJ. Identification and characterization of the human myeloperoxidase promoter. Leukemia. 1995; 9(5): 848-857.

10. Zhao W-G, Regmi A, Austin ED, Braun JE, Racine M, Austin GE. Cis-elements in the promoter region of the human myeloperoxidase gene. Leukemia. 1996; 10(7): 1089-1103.

11. Zhao W-G, Lu J-P, Austin GE. Enhancement of myeloperoxidase promoter function by PEBP2/CBF or inhibition by an Sp1-containing repressor requires the participation of a third element DP4. Blood. 1996; 88 Suppl 1:47a.

12. Zhao W-G, Regmi A, Lu J-P, Austin GE. Identification and functional analysis of multiple murine myeloperoxidase (MPO) promoters and comparison with the human MPO promoter region. Leukemia 1997; 11(1): 97-105.

13. Austin GE, Zhao WG, Regmi A, Lu JP, Braun J. identification of an upstream enhancer containing an AML1 site in the human myeloperoxidase (MPO) gene. Leuk Res. 1998; 22(11): 1037-1048.

14. Yao C, Qin Z, Works KN, Austin GE, Young AN. C/EBP and C-Myb sites are

important for the functional activity of the human myeloperoxidase upstream enhancer. Biochem Biophys Res Commun. 2008; 371(2): 309-314.

15. Khoury H, Dalal BI, Nantel SH, Horsman DE, Lavoie JC, Shepherd JD, et al. Correlation between karyotype and quantitative immunophenotype in acute myelogenous leukemia with t(8;21). Modern Pathol. 2004; 17(10): 1211-1216.

16. Shimada H, Ichikawa H, Ohki M. Potential involvement of the AML1-MTG8 fusion protein in the granulocytic maturation characteristic of the t(8;21) acute myelogenous leukemia revealed by microarray analysis. Leukemia. 2002; 16(5): 874-885.

17. Meyers S, Lenny N, Hiebert SW. The t(8;21) fusion protein interferes with AML-1B-dependent transcriptional activation. Mol Cell Biol. 1995; 15(4): 1974-1982.

18. Tominaga-Sato S, Tsushima H, Ando K, Itonaga H, Imaizumi Y, Imanishi D, et al. Expression of myeloperoxidase and gene mutations in AML patients with normal karyotype: double CEBPA mutations are associated with high percentage of MPO positivity in leukemic blasts. Int J Hematol. 2011; 94(1): 81-89.

19. Kato N, Kitamura J, Doki N, Komeno Y, Watanabe-Okochi N, Togami K, et al. Two types of C/EBPα mutations play distinct but collaborative roles in leukemogenesis; lessons from clinical data and BMT models. Blood. 2011; 117(1): 221-233.

20. Momparler RL, Bovenzi V. DNA methylation and cancer. J Cell Physiol. 2000;

183(2): 145-154.

21. Issa JP. Methylation and prognosis: of molecular clocks and hypermethylation phenotypes. Clin Cancer Res. 2003; 9(8): 2879-2881.

22. Jones PA, Baylin SB. The epigenetic of cancer. Cell. 2007; 128(4):683-692.

23. Schmelz K, Sattler N, Wagner M, Lubbert M, Dorken B, Tamm I. Induction of gene expression by 5-Aza-2'-deoxycytidine in acute myeloid leukemia (AML) and myelodysplastic syndrome (MDS) but not epithelial cells by DNA-methylation-dependent and –independent mechanisms. Leukemia. 2005; 19(1): 103-111.

24. Figueroa ME, Lugthart S, Li Y, Erpelinck-Verschueren C, Deng X, Christos PJ, et al. DNA methylation signatures identify biologically distinct subtypes in acute myeloid leukemia. Cancer Cell. 2010; 17(1): 13-27.

25. Bibikova M, Le J, Barnes B, Saedinia-Melnyk S, Zhou L, Shen R, et al. Genome-wide DNA methylation profiling using infinium® assay. Epigenomics 2009;1(1):177-200.

26. Gunderson KL, Steemers FJ, Ren H, Ng P, Zhou L, Tsan C, et al. Whole-genome genotyping. Methods Enzymol. 2006;410:359-376.

27. Steemers FJ, Gunderson KL. Whole genome genotyping technologies on the

BeadArray platform. Biotechnol. J. 2007;2(1):41-49.

28. Yang AS, Estecio MR, Doshi K, Kondo Y, Tajara EH, Issa JP. A simple method for estimating global DNA methylation using bisulfite PCR of repetitive DNA elements. Nucleic Acids Res. 2004;32(3):e38.

29. Okano M, Xie S, Li E. Cloning and characterization of a family of novel mammalian DNA (cytosine-5) methyltransferases. Nat Genet. 1998; 19(3): 219-220.

30. Aimiuwu J, Wang H, Chen P, Xie Z, Wang J, Liu S, et al. RNA-dependent inhibition of ribonucleotide reductase is a major pathway for 5-azacitidine activity in acute myeloid leukemia. Blood. 2012; 119(22): 5229-5238.

31. Aoyama S, Nakano H, Danbara M, Higashihara M, Harigae H, Takahashi S. The differentiation and apoptotic effects of 2-aza-5'deoxycytidine are dependent on the PU.1 expression level in PU.1-transgenic K562 cells. Biochem Biophys Res Commun. 2012; 420(4): 775-781.

32. Curik N, Burda P, Vargova K, Pospisil V, Belickova M, Vlckova P, et al. 5-azacitidine in aggressive myelodysplastic syndromes regulates chromatin structure at PU.1 gene and cell differentiation capacity. Leukemia. 2012; 26(8): 1804-1811.

33. Kulis M, Heath S, Bibikova M, Queiros AC, Navarro A, Clot G, et al. Epigenomic analysis detects widespread gene-body DNA hypomethylation in chronic lymphocytic

leukemia. Nat Genet. 2012; 44(11): 1236-1242.

34. Razin A, Riggs AD. DNA methylation and gene function. Science. 1980; 210(4470): 604-610.

35. Robertson KD. DNA methylation, methyltransferases, and cancer. Oncogene. 2001; 20(24): 3139-3155.

36. Arand J, Spieler D, Karius T, Branco MR, Meilinger D, Meissner A, et al. In vitro control of CpG and non-CpG DNA methylation by DNA methyltransferases. PLoS Genet. 2012; 8(6): e1002750.

37. Ley TJ, Ding L, Walter MJ, McLellan MD, Lamprecht T, Larson DE, et al. DNMT3A mutations in acute myeloid leukemia. N Engl J Med. 2010; 363(25): 2424-2433.

38. Marcucci G, Metzeler KH, Schwind S, Becker H, Maharry K, Mrozek K, et al. Age-related prognostic impact of different types of DNMT3A mutations in adults with primary cytogenetically normal acute myeloid leukemia. J Clin Oncol. 2012; 30(7): 742-750.

39. Yan XJ, Xu J, Gu ZH, Pan CM, Lu G, Shen Y, et al. Exome sequencing identifies somatic mutations of DNA methyltransferase gene DNMT3A in acute monocytic leukemia. Nat Genet. 2011; 43(4): 309-315.

40. Yamashita Y, Yuan J, Suetake I, Suzuki H, Ishikawa Y, Choi YL, et al. Array-based genomic resequencing of human leukemia. Oncogene. 2010; 29(25): 3723-3731.

41. Kim SJ, Zhao H, Hardikar S, Singh AK, Goodell MA, Chen T. A DNMT3A mutation common in AML exhibits dominant-negative effects in murine ES cells. Blood. 2013: doi:10.1182/blood-2013-02-483487.

42. Thol F, Damm F, Ludeking A, Winschel C, Wagner K, Morgan M, et al. Incidence and prognostic influence of DNMT3A mutations in acute myeloid leukemia. J Clin Oncol. 2011;29(21):2889-2896.

43. Robertson KD, Uzvolgyi E, Liang G, Talmadge C, Sumegi J, Gonzales FA, et al. The human DNA methyltransferase (DNMTs) 1, 3a and 3b: coordinate mRNA expression in normal tissue and overexpression in tumors. Nucleic Acids Res. 1999: 27(11); 2291-2298.

44. Girault I, Tozlu S, Lidereau R, Bieche I. Expression analysis of DNA methyltransferase 1, 3A, and 3B in sporadic breast carcinomas. Clin Cancer Res. 2003; 9(12): 4415-4422.

45. Roll JD, Rivenbark AG, Jones WD, Coleman WB. DNMT3b overexpression contributes to a hypermethylator phenotype in human breast cancer cell lines. Mol Cancer. 2008; 7: 15.

46. Mizuno S, Chijiwa T, Okamura T, Akashi K, Fukumaki Y, Niho Y, et al. Expression of DNA methyltransferases DNMT1, 3A, and 3B in normal hematopoiesis and in acute and chronic myelogenous leukemia. Blood. 2001; 97(5): 1172-1179.

47. Hayette S, Thomas X, Jallades L, Chabane K, Charlot C, Tigaud I, et al. High DNA methyltransferase DNMT3B levels: a poor prognostic marker in acute myeloid leukemia. PLoS One. 2012; 7(12): e51527.

48. Shah MY, Vasanthankumar A, Barnes NY, Figueroa ME, Kamp A, Hendrick C, et al. DNMT3B7, a truncated DNMT3B isoform expressed in human tumors, disrupts embryonic development and accelerates lymphomagenesis. Cancer Res. 2010; 70(14): 5840-5850.

49. Gopalakrishnan S, Van Emburgh BO, Shan J, Su Z, Fields CR, Vieweg J, et al. A novel DNMT3B splice variant expressed in tumor and pluripotent cells modulates genomic DNA methylation patterns and displays altered DNA binding. Mol Cancer Rs. 2009; 7(10): 1622-1634.

50. Van Emburgh BO, Robertson KD. Modulation of DNMT3b function in vitro by interactions with Dnmt3L, Dnmt3a and Dnmt3b splice variants. Nucleic Acids Res. 2001; 39(12): 4984-5002.

51. Gopalakrishna-Pilai S, Iverson LE. A DNMT3B alternatively spliced exon and

encoded peptide are novel biomarkers of human pluripotent stem cells. PLoS One. 2011; 6(6): e20663.

52. Wu CH, Gordon J, Rastegar M, Ogretmen B, Safa AR. Proteinase-3, a serine protease which mediates doxorubicin-induced apoptosis in the HL-60 leukemia cell line, is downregulated in its doxorubicin-resistant variant. Oncogene. 2002; 21(33): 5160-5174.

53. Aqirre X, Roman-Gomez J, Jimenez-Velasco A, Garate L, Montiel-Duarte C, Navarro G, et al. ASPP1, a common activator of TP53, is inactivated by aberrant methylation of its promoter in acute lymphoblastic leukemia. Oncogene. 2006; 25(13): 1862-1870.

54. Fazi F, Rosa A, Fatica A, Gelmetti V, De Marchis ML, Nervi C, et al. A minicircuitry comprised of microRNA-223 and transcription factors NFI-A and C/EBPalpha regulates human granulopoiesis. Cell. 2005; 123(5): 819-831.

55. Taketani T, Taki T, Shibuya N, Kikuchi A, Hanada R, Hayashi Y. Novel NUP98-HOXC11 fusion gene resulted from a chromosomal break within exon 1 of HOXC11 in acute myeloid leukemia with t(11;12)(p15;q13). Cancer Res. 2002; 62(16): 4571-4574.

56. Lin M, Sutherland DR, Horsfall W, Totty N, Yeo E, Nayar R, et al. Cell surface

antigen CD109 is a novel member of the alpha(2) macroglobulin/C3, C4, C5 family of thioester-containing proteins. Blood. 2002; 99(5): 1683-1691.

57. Delhommeau F, Dupont S, Della Valle V, James C, Trannoy S, Masse A, et al. Mutation in TET2 in myeloid cancers. N Engl J Med. 2009; 360(22): 2289-2301.

58. Mardis ER, Ding L, Dooling DJ, Larson DE, McLellan MD, Chen K, et al. Recurring mutations found by sequencing an acute myeloid leukemia genome. N Engl J Med. 2009; 361(11): 1058-1066.

59. Carbuccia N, Trouplin V, Gelsi-Boyer V, Murati A, Rocquain J, Adelaide J, et al. Mutuali exclusion of ASXL1 and NPM1 mutations in a series of acute myeloid leukemias. Leukemia. 2010; 24(2): 469-473.

60. Shih AH, Abdel-Wahab O, Patel JP, Levine RL. The role of mutations in epigenetic regulators in myeloid malignancies. Nat Rev Cancer. 2012; 12(9): 599-612.

Figure 1

Expression of MPO mRNA in MPOa-L AML cell lines after the DNMT-i treatment.

The expression of MPO mRNA in each cell line cultured with or without DNMT-i was analyzed by quantitative reverse-transcriptase PCR (QRT-PCR). Results are expressed as the mean and SD of three independent studies (*$P < 0.05$).

Figure 2

The significant relationship between the percentage of MPO-positive myeloblasts in bone marrow smears and the expression level of MPO mRNA in CD34-positive AML cells.

The percentage of MPO-positive myeloblasts in bone marrow smears (estimated using the diaminobenzidine method) showed a significant relationship with the amount of MPO mRNA (by QRT-PCR) in CD34-positive AML cells ($P < 0.001$).

Figure 3

DNA methylation status of the *MPO* gene promoter and its gene body in CD34-positive AML cells.

Quantification of DNA methylation in 13 CpG sites was performed using the Illumina infinium assay (color-coded as indicated by the scale at the left). The positions of the CpG sites (yellow square), CpG islands (blue square), and exons (purplish square) evaluated were from the NCBI database. The green arrow indicates the transcription factor binding sites (based on the TRANSFAC database).

Figure 4

(a) Outline of the steps used to build the DNA methylation classifier.

(b) Heatmap of the aberrant DNA methylation signature in CD34-positive cells obtained from MPOa-H AML.

Data of MPOa-H AML patients were compared with those from MPOa-L AML and Ph+ALL patients and healthy donors. The gene symbols are indicated at the left.

Figure 5

(a) Relationship between *MPO* and *DNMT3B* gene expression.

A significant inverse relationship was observed between the amounts of MPO mRNA and DNMT3B mRNA in CD34-positive cells obtained from AML patients. Analysis for the relative expression level of DNMT3B mRNA was performed using QRT-PCR.

(b) Relationship between *MPO* and *DNMT3B* gene expression in AML with a normal karyotype.

A significant inverse relationship was observed between the amount of MPO mRNA and DNMT3B mRNA in CD34-positive cells obtained from AML patients with a normal karyotype.

Figure 1

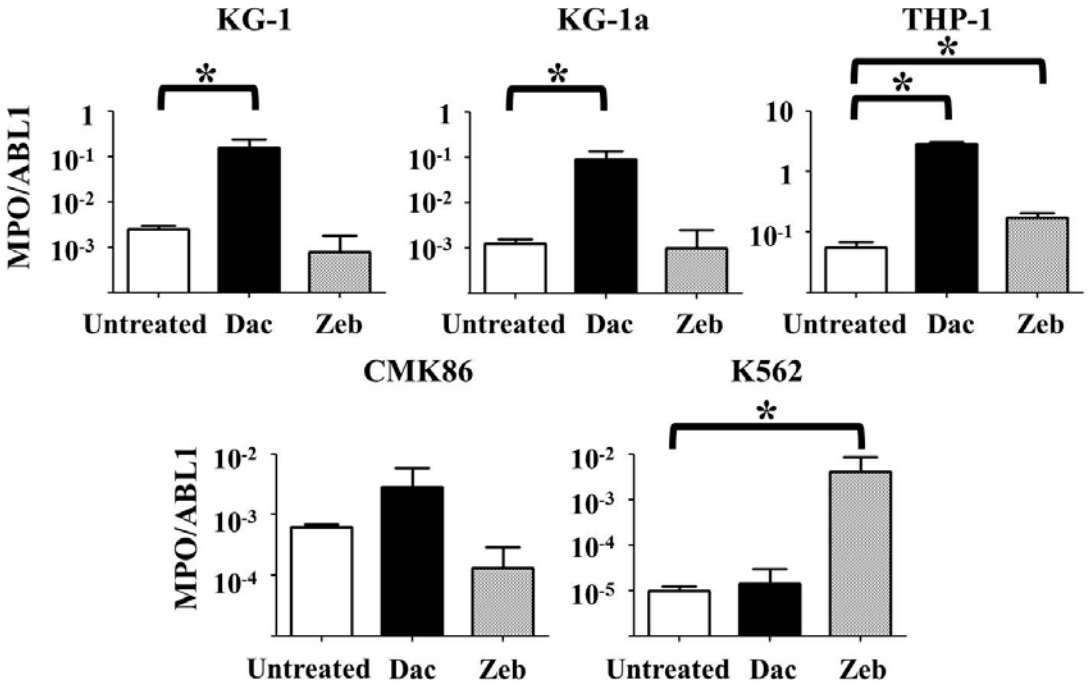


Figure 2

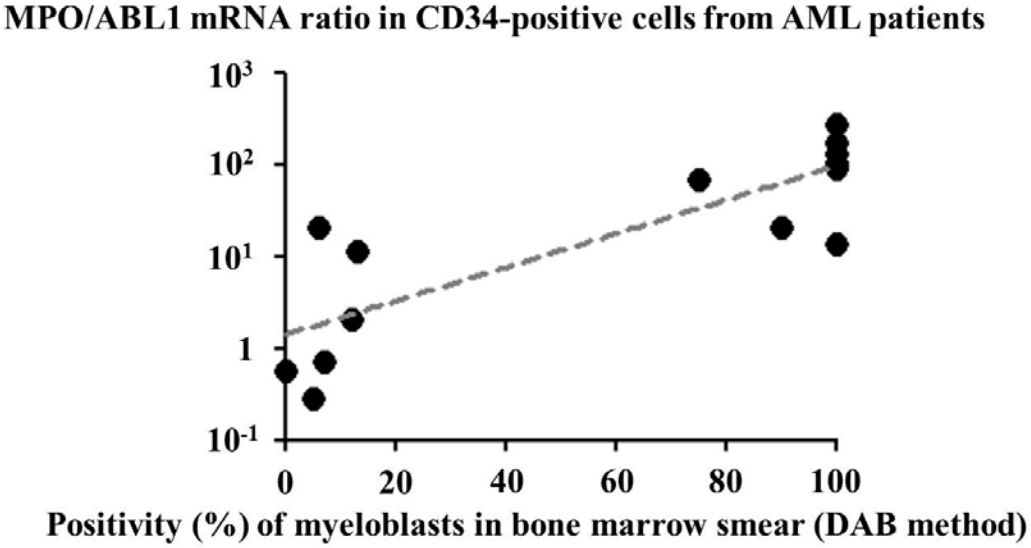


Figure 3

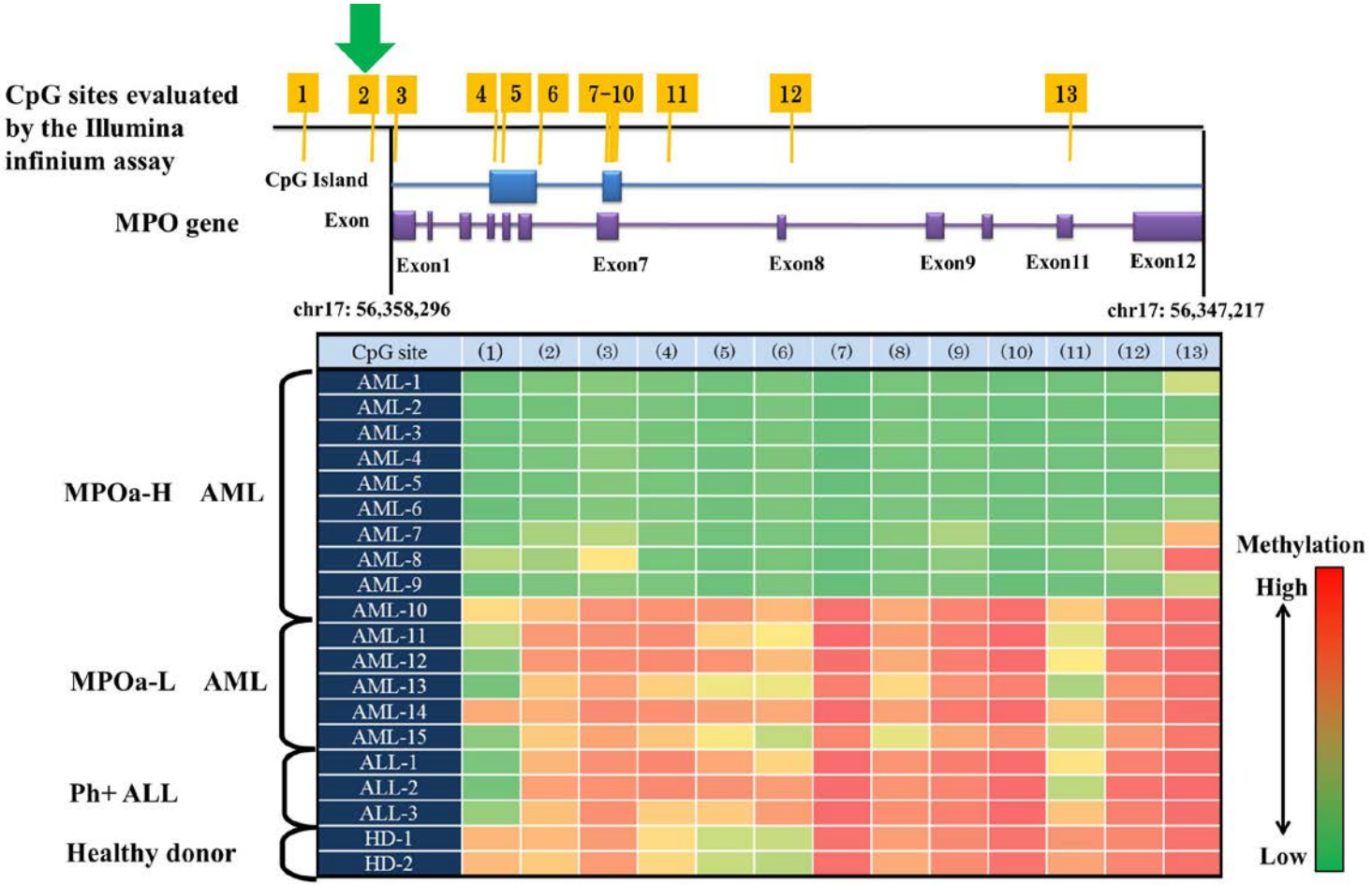
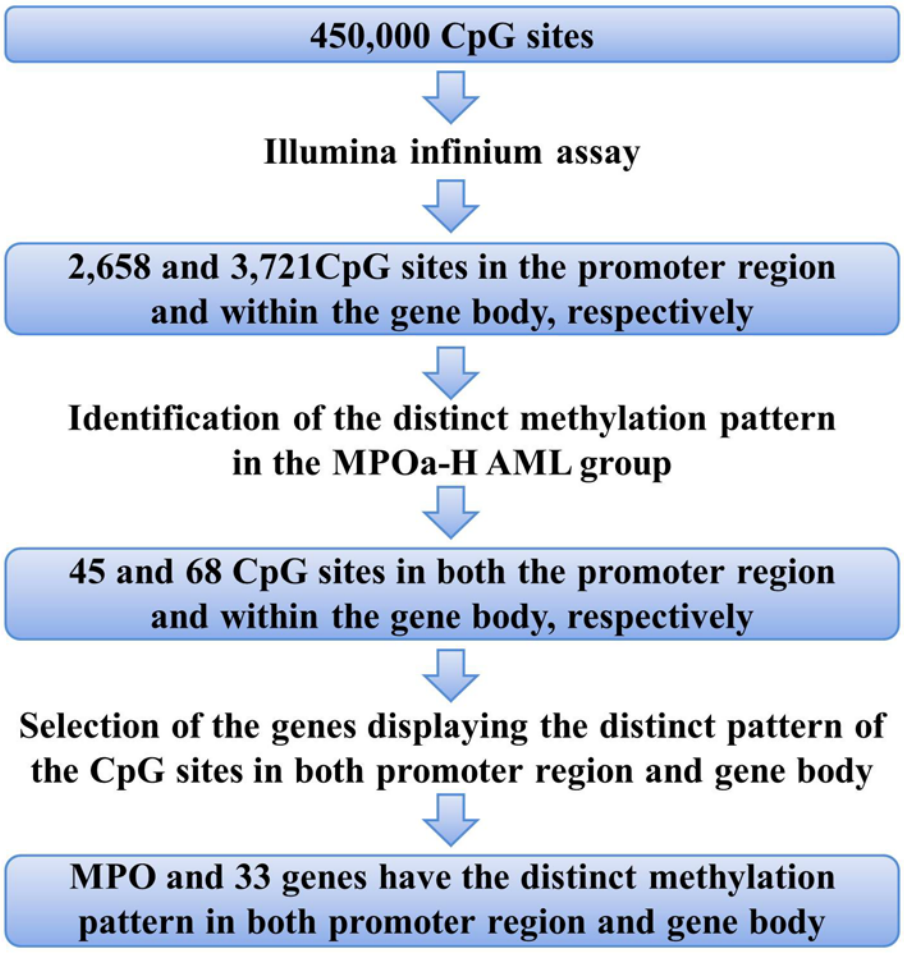

CpG sites evaluated by the Illumina infinium assay
1
2
3
4
5
6
7-10
11
12
13
CpG Island
MPO gene
Exon
Exon1
Exon7
Exon8
Exon9
Exon11
Exon12
chr17: 56,358,296
chr17: 56,347,217
CpG site
(1)
(2)
(3)
(4)
(5)
(6)
(7)
(8)
(9)
(10)
(11)
(12)
(13)
AML-1
AML-2
AML-3
AML-4
AML-5
AML-6
AML-7
AML-8
AML-9
AML-10
AML-11
AML-12
AML-13
AML-14
AML-15
ALL-1
ALL-2
ALL-3
HD-1
HD-2
MPOa-H AML
MPOa-L AML
Ph+ ALL
Healthy donor
Methylation
High
Low

Figure 4

a


450,000 CpG sites
Illumina infinium assay
2,658 and 3,721CpG sites in the promoter region and within the gene body, respectively
Identification of the distinct methylation pattern in the MPOa-H AML group
45 and 68 CpG sites in both the promoter region and within the gene body, respectively
Selection of the genes displaying the distinct pattern of the CpG sites in both promoter region and gene body
MPO and 33 genes have the distinct methylation pattern in both promoter region and gene body

b

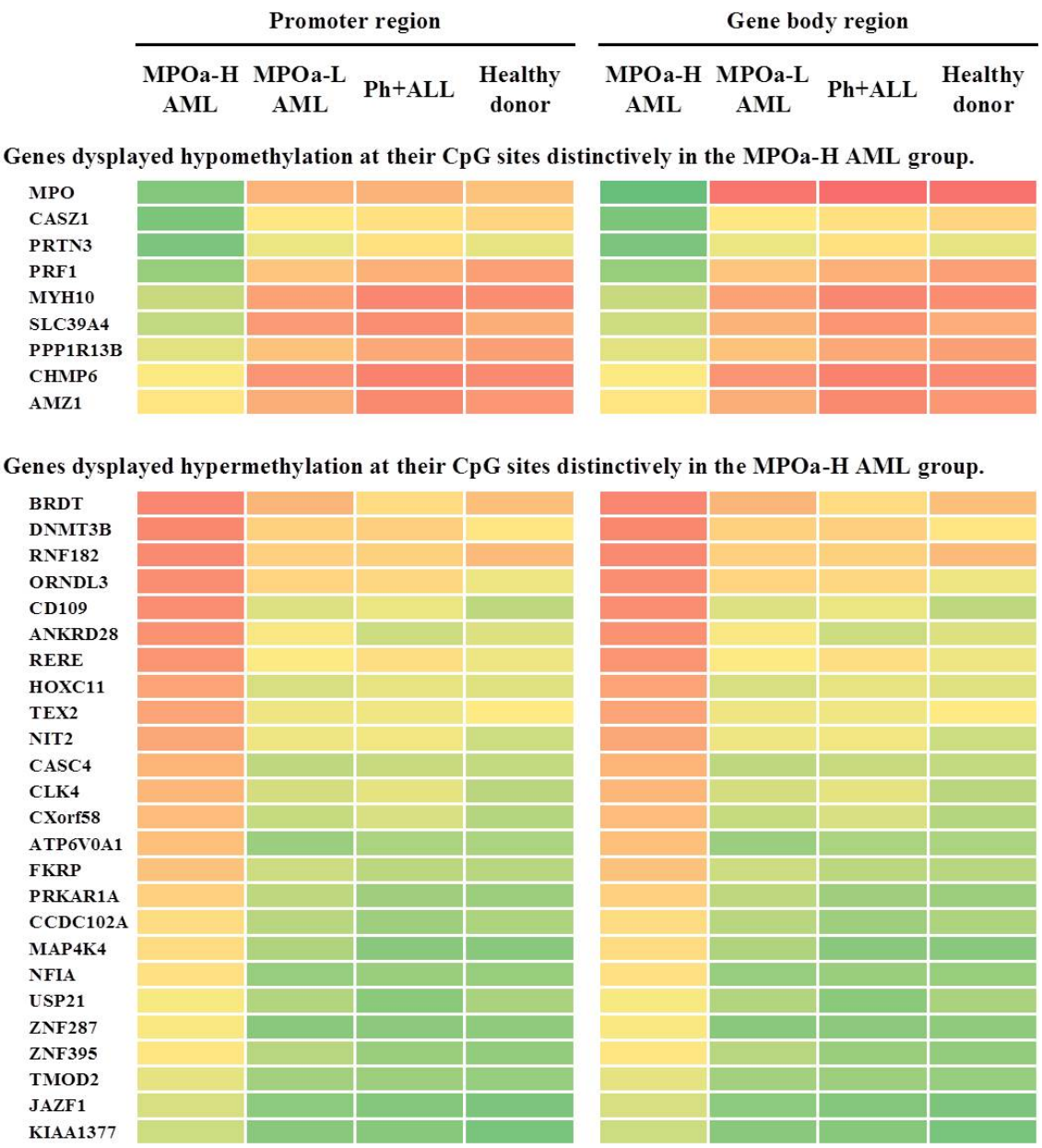
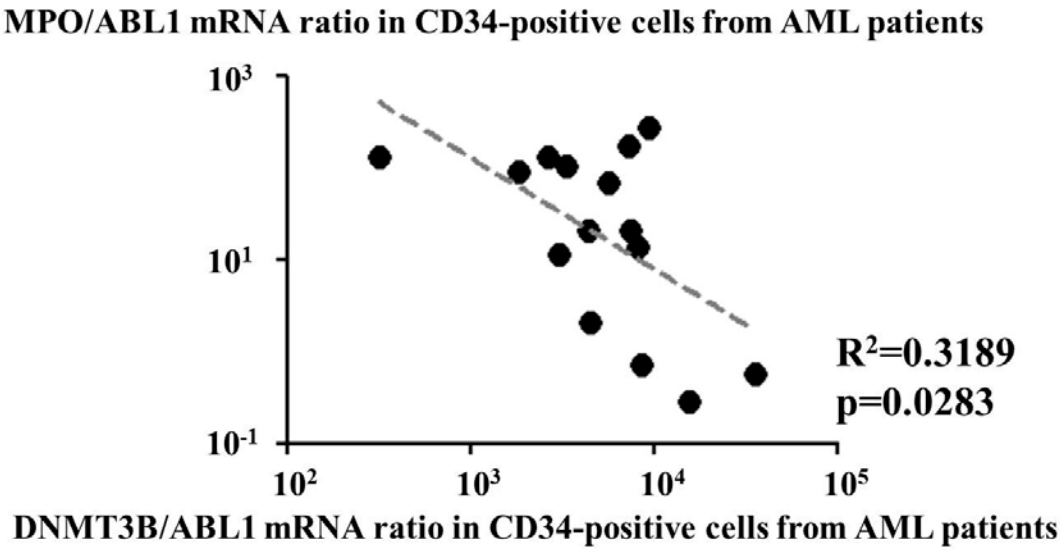

Promoter region
Gene body region
MPOa-H AML
MPOa-L AML
Ph+ALL
Healthy donor
MPOa-H AML
MPOa-L AML
Ph+ALL
Healthy donor
Genes dysplayed hypomethylation at their CpG sites distinctively in the MPOa-H AML group.
MPO
CASZ1
PRTN3
PRF1
MYH10
SLC39A4
PPP1R13B
CHMP6
AMZ1
Genes dysplayed hypermethylation at their CpG sites distinctively in the MPOa-H AML group.
BRDT
DNMT3B
RNF182
ORNDL3
CD109
ANKRD28
RERE
HOXC11
TEX2
NIT2
CASC4
CLK4
CXorf58
ATP6V0A1
FKRP
PRKAR1A
CCDC102A
MAP4K4
NFIA
USP21
ZNF287
ZNF395
TMOD2
JAZF1
KIAA1377

Figure 5

a


MPO/ABL1 mRNA ratio in CD34-positive cells from AML patients
10³
10¹
10⁻¹
10²
10³
10⁴
10⁵
R²=0.3189
p=0.0283
DNMT3B/ABL1 mRNA ratio in CD34-positive cells from AML patients

b

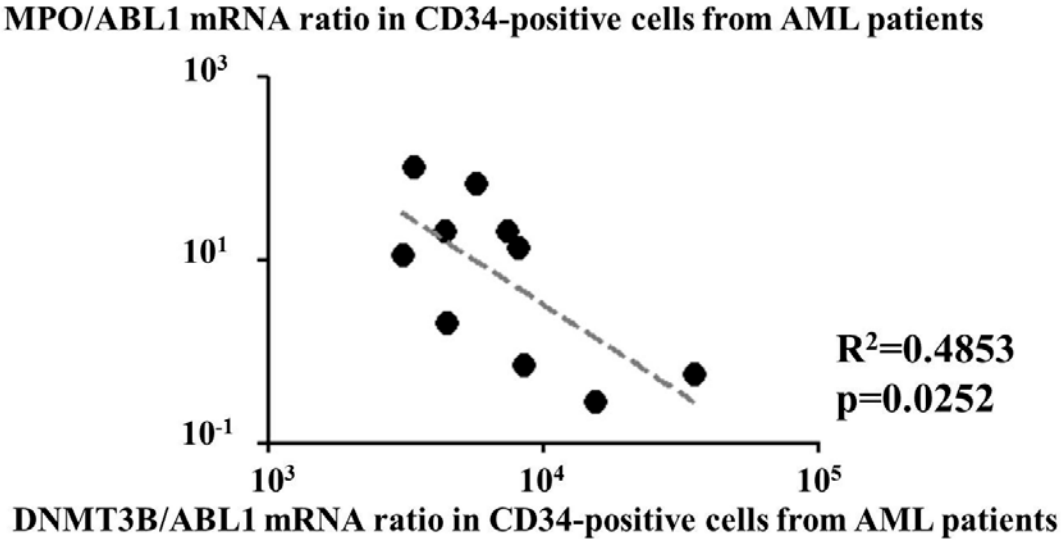

MPO/ABL1 mRNA ratio in CD34-positive cells from AML patients
10³
10¹
10⁻¹
10³
10⁴
10⁵
R²=0.4853
p=0.0252
DNMT3B/ABL1 mRNA ratio in CD34-positive cells from AML patients

Table 1 Expression of MPO and *FLT3*, *NPM1*, and *CEBPA* Mutations in clinical samples

UPN	Sex	Disease type (FAB)	Karyotype	Fusion gene	MPO positivity in bone marrow smears	MPO positivity of CD34-positive cells	MPO/ABL1 mRNA ratio	*FLT3-ITD*	*NPM1*	*CEBPA*
1	M	AML (M1)	t(8;21)	*RUNX1-RUNX1T1*	100%	100%	173.068	Negative	wt	wt
2	M	AML (M2)	t(8;21)	*RUNX1-RUNX1T1*	100%	100%	129.077	Negative	wt	wt
3	M	AML (M2)	t(8;21)	*RUNX1-RUNX1T1*	100%	100%	90.827	Positive	wt	wt
4	M	AML (M4)	inv(16)	*CBFB-MYH11*	100%	95%	272.320	Negative	wt	Single mutation
5	M	AML (M4)	inv(16)	*CBFB-MYH11*	100%	100%	127.669	Negative	wt	wt
6	F	AML (M1)	NC	-	100%	100%	807.332	Negative	wt	Double mutation
7	F	AML (M1)	NC	-	100%	100%	13.830	Negative	wt	Double mutation
8	M	AML (M2)	NC	-	75%	70%	104.525	Negative	wt	wt
9	M	AML (M2)	NC	-	90%	90%	69.408	Negative	wt	Double mutation
10	F	AML (M4)	NC	-	6%	6%	20.163	Negative	wt	wt
11	M	AML (M5a)	NC	-	0%	0%	0.559	Negative	wt	wt
12	M	AML (M1)	NC	-	5%	5%	0.287	Positive	Mutation (type A)	wt
13	F	AML (M5b)	NC	-	7%	8%	0.724	Positive	Mutation (type A)	wt
14	M	AML (M2)	NC	-	13%	2%	11.206	Positive	wt	wt
15	M	AML (M5b)	NC	-	12%	0%	2.065	Positive	Mutation (type G)	wt
16	F	ALL (L2)	t(9;22)	*minor BCR-ABL1*	0%	0%	0.207	Negative	wt	wt
17	F	ALL (L2)	t(9;22)	*minor BCR-ABL1*	0%	0%	0.174	Negative	wt	wt
18	F	ALL (L2)	t(9;22)	*minor BCR-ABL1*	0%	0%	0.124	Negative	wt	wt
19	M	Healthy donor	NC	-	-	6%	31.858	Negative	wt	wt
20	F	Healthy donor	NC	-	-	9%	37.612	Negative	wt	wt

Abbreviations: UPN represents unique patient number; FAB, French-American-British classifications; M, male; F, female; AML, acute myeloid leukemia; ALL, acute lymphoid leukemia; NC, normal karyotype.

Quantitative analysis of MPO and ABL1 mRNA was performed by quantitative reverse-transcriptional PCR amplifications after purifying CD34-positive cells. Mutation analysis of *FLT3*, *NPM1*, and *CEBPA* was the same as that in the cell lines. MPO positivity was assessed in bone marrow smears by the percentage of MPO-positive blasts in MPO-stained bone marrow smears with the diaminobenzidine method. The MPO positivity of CD34-positive cells was also evaluated in cytospin slides.