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

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Expression of myeloperoxidase in acute myeloid leukemia blasts mirrors the distinct DNA methylation pattern involving the downregulation of DNA methyltransferase \textit{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 \textit{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 RUNXI-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 \textit{MPO} transcription in AML blasts. These contradictions suggest that the regulation of \textit{MPO} gene transcription in leukemia cells differs from that in normal hematopoietic cells, and also that the control of \textit{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 \textit{MPO} [23]. However, because \textit{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 Zellkulturen (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 ($\beta = \frac{\text{intensity of the Methylated allele (M)}}{\text{(intensity of the Unmethylated allele (U) + intensity of the Methylated allele (M)) + 100}}$).

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 [\leq50%] 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 \textit{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, (\textit{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 (\textit{p}<0.0001. Figure 2, and \textit{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 \textit{MPO} gene correlated with the expression of both the \textit{MPO} gene and its enzymatic activity in CD34-positive cells from clinical samples
To determine the methylation status of the \textit{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 \textit{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 \textit{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 \textit{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 \textit{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 \textit{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 \textit{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. R²=0.3189, p=0.0283). This relationship was also found in 10 AML patients with a normal karyotype out of these 15 AML patients (R²=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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**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

MPO/ABL1 ratio in different cell lines:

- **KG-1**
  - Untreated: 10^{-3}
  - Dac: 10^{-1}
  - Zeb: 10^{-2}

- **KG-1a**
  - Untreated: 10^{-3}
  - Dac: 10^{-1}
  - Zeb: 10^{-2}

- **THP-1**
  - Untreated: 10^{-1}
  - Dac: 10^{-2}
  - Zeb: 10^{-3}

Figure 2

MPO/ABL1 mRNA ratio in CD34-positive cells from AML patients:

Positivity (%) of myeloblasts in bone marrow smear (DAB method)
Figure 3

CpG sites evaluated by the Illumina infinium assay

MPO gene

Exon 1  Exon 2  Exon 3  Exon 4  Exon 5  Exon 6  Exon 7  Exon 8  Exon 9  Exon 10  Exon 11  Exon 12

chr17: 56,358,296  chr17: 56,347,217

MPOa-H  AML

MPOa-L  AML

Ph+ ALL

Healthy donor

Methylation

High

Low
Figure 4

450,000 CpG sites →

Illumina infinium assay →

2,658 and 3,721 CpG 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
Genes dysplayed hypomethylation at their CpG sites distinctively in the MPOa-H AML group.

<table>
<thead>
<tr>
<th>Genes</th>
<th>MPOa-H AML</th>
<th>MPOa-L AML</th>
<th>Ph+ALL</th>
<th>Healthy donor</th>
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Genes dysplayed hypermethylation at their CpG sites distinctively in the MPOa-H AML group.

<table>
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<tr>
<th>Genes</th>
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<th>MPOa-L AML</th>
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<th>Healthy donor</th>
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</table>
Figure 5

a

MPO/ABL1 mRNA ratio in CD34-positive cells from AML patients

![Graph showing MPO/ABL1 mRNA ratio with regression line and R^2 and p-value values]

DNMT3B/ABL1 mRNA ratio in CD34-positive cells from AML patients

b

MPO/ABL1 mRNA ratio in CD34-positive cells from AML patients

![Graph showing MPO/ABL1 mRNA ratio with regression line and R^2 and p-value values]

DNMT3B/ABL1 mRNA ratio in CD34-positive cells from AML patients
<table>
<thead>
<tr>
<th>UPN</th>
<th>Sex</th>
<th>Disease type (FAB)</th>
<th>Karyotype</th>
<th>Fusion gene</th>
<th>MPO positivity in bone marrow smears</th>
<th>MPO positivity of CD34-positive cells</th>
<th>MPO/ABL1 mRNA ratio</th>
<th>FLT3-ITD</th>
<th>NPM1</th>
<th>CEBPA</th>
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</thead>
<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>AML (M1)</td>
<td>t(8;21)</td>
<td>RUNXI-RUNXIT1</td>
<td>100%</td>
<td>100%</td>
<td>173.068</td>
<td>Negative</td>
<td>wt</td>
<td>wt</td>
</tr>
<tr>
<td>2</td>
<td>M</td>
<td>AML (M2)</td>
<td>t(8;21)</td>
<td>RUNXI-RUNXIT1</td>
<td>100%</td>
<td>100%</td>
<td>129.077</td>
<td>Negative</td>
<td>wt</td>
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<td>RUNXI-RUNXIT1</td>
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<td>100%</td>
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<td>Positive</td>
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<tr>
<td>4</td>
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<td>AML (M4)</td>
<td>inv(16)</td>
<td>CBFB-MYH11</td>
<td>100%</td>
<td>95%</td>
<td>272.320</td>
<td>Negative</td>
<td>wt</td>
<td>Single mutation</td>
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<td>5</td>
<td>M</td>
<td>AML (M4)</td>
<td>inv(16)</td>
<td>CBFB-MYH11</td>
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<td>100%</td>
<td>127.669</td>
<td>Negative</td>
<td>wt</td>
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<td>807.332</td>
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<td>13.830</td>
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<td>70%</td>
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<td>9</td>
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<td>wt</td>
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<td>AML (M5b)</td>
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<td>12%</td>
<td>0%</td>
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<td>Positive</td>
<td>wt</td>
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<td>0%</td>
<td>0.207</td>
<td>Negative</td>
<td>wt</td>
<td>wt</td>
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<td>minor BCR-ABL1</td>
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<td>0%</td>
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<td>wt</td>
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<tr>
<td>18</td>
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<td>t(9;22)</td>
<td>minor BCR-ABL1</td>
<td>0%</td>
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<td>wt</td>
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<td>37.612</td>
<td>Negative</td>
<td>wt</td>
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</table>
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.