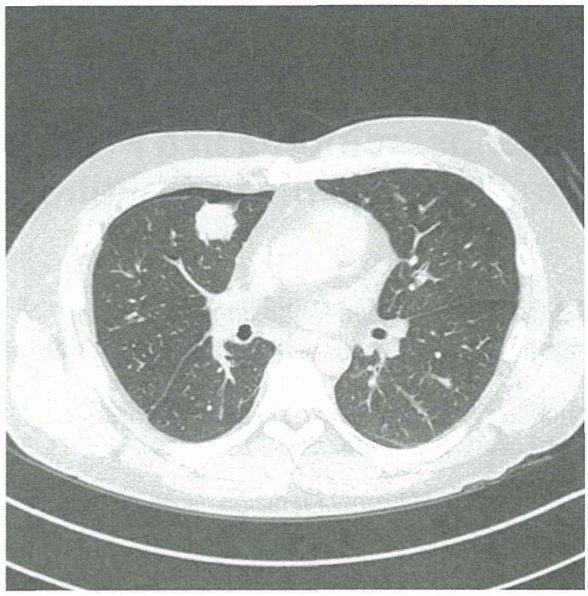

Figure 1. Chest computed tomography scan after thoracentesis. A nodule with a diameter of ~3 cm is detected in the right upper lobe.

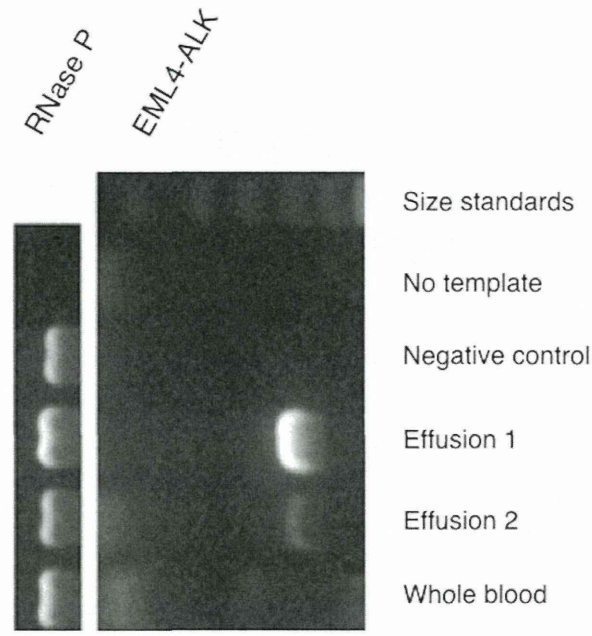

Figure 2. Gel electrophoresis of RT-PCR products: Multiplex RT-PCR was conducted on pleural effusion premixed with RLT buffer (Qiagen, Hilden, Germany) (Effusion 1), a frozen aliquot of the original effusion (Effusion 2), and a frozen aliquot of whole peripheral blood. The same experiment was also conducted with no template or an *EML4–ALK*-negative specimen (Negative control). For the internal control of RT-PCR, the human ribonuclease P (RNase P) cDNA was also amplified. Size standards (50 bp DNA ladder, Life Technologies Corporation, Carlsbad, CA).

with ALL and enrolled in a clinical trial for juvenile high-risk ALL (10). He had been treated with the AL851 regimen, receiving vincristine, prednisolone, daunorubicin, l-asparaginase, methotrexate, 6-mercaptopurine, doxorubicin, cytosine arabinoside, 6-mercaptopurine, and cyclophosphamide. Cranial irradiation was used to prevent central nervous system leukemia; however, thoracic irradiation was not performed (10). Complete remission was achieved.

Considering his medical history, he was found to have a secondary lung adenocarcinoma at an advanced stage. Mutation analysis of mononuclear cells in his pleural fluid indicated that his tumor carried the wild-type epidermal growth factor receptor gene (*EGFR*). After pleurodesis, treatment with carboplatin and paclitaxel with bevacizumab was started. During this first-line treatment, it was suspected that his lung adenocarcinoma could be an *EML4–ALK*-positive tumor, given the fact that he was young and his tumor was negative for *EGFR* mutations. Thus, the multiplex reverse-transcription polymerase chain reaction (RT-PCR) was performed to test for *EML4–ALK* fusion complementary DNA (cDNA) in his tumor cells (Forward primer; 5′-GTGCAG TGTTTAGCATTCTTGGGG-3′ and reverse primer; 5′-TCTT GCCAGCAAAGCAGTAGTTGG-3′) (11). As shown in Fig. 2, RT-PCR showed that his tumor did indeed carry *EML4–ALK*. Nucleotide sequencing of this PCR product further confirmed the presence of *EML4–ALK* variant 1 cDNA in which exon 13 of *EML4* was fused in-frame to exon 20 of *ALK* (Fig. 3). These data suggest that the patient had a secondary *EML4–ALK*-positive NSCLC.

After four cycles of carboplatin and paclitaxel with bevacizumab and two cycles of bevacizumab maintenance treatment, his tumor progressed. He was then treated with crizotinib, an ALK-specific inhibitor. The tumor initially responded to crizotinib, but became refractory after several months of treatment. After that, several chemotherapy regimens, including pemetrexed, cisplatin plus docetaxel, gemcitabine and TS-1, were administered; however, all were ineffective. He died almost 2 years after the diagnosis of lung cancer.

DISCUSSION

This patient had been diagnosed with and treated for ALL almost 20 years earlier and subsequently diagnosed with lung adenocarcinoma. It is widely recognized that the risk of secondary malignancies increases as childhood-cancer survivors progress through adulthood (1–6). There is no widely accepted definition of secondary malignant neoplasm; however, considering the definition of the Childhood Cancer Survivor Study (CCSS) (1), we concluded that his lung adenocarcinoma was a secondary malignant neoplasm. In the CCSS report, the most frequent secondary epithelial malignancies occurring ≥5 years after childhood cancer were breast and thyroid cancer. However, bronchial and lung cancers were also epithelial malignancies whose subsequent risks were elevated. The 30-year cumulative incidence for selected subsequent cancers was 0.1% for lung cancer, and the standardized incidence ratio (SIR) of second bronchial and lung cancers was 3.4 (95% confidence interval [CI] = 1.9–6.1) (1).

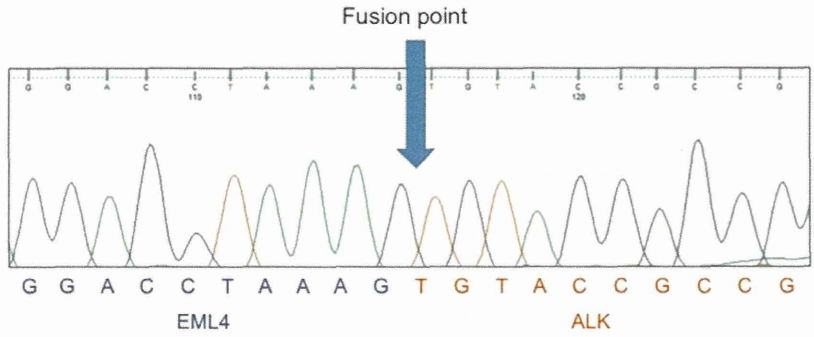

Figure 3. The nucleotide sequence of the EML4–ALK variant1 cDNA isolated from this patient is shown. Genome nucleotides corresponding to EML4 or to ALK are shown in blue and red, respectively. An arrowhead indicates the fusion point.

Recurrent chromosome translocations were found in most cases of secondary hematological malignancies after childhood ALL treated with Berlin-Frankfurt-Munster (BFM)-based regimens (8). Several anti-cancer drugs are considered to be potent clastogenic agents. Thus, secondary hematological malignancies may result from illegitimate recombination of chromosomal fragments caused by such anti-cancer agents (7). In this regard, BFM-based treatment might play a direct role in chromosome translocations detected in secondary malignancies. On the other hand, oncogenic driver mutations, such as *EGFR* mutant or *K-ras* mutant, have not been reported in relation to secondary lung cancer so far. This case also did not harbor any *EGFR* mutations. In this regard, oncogenic point mutations in lung cancer may have only a weak association with carcinogenicity of anti-cancer drugs.

It was previously accepted that recurrent chromosome translocations play a major role in the molecular pathogenesis of hematological malignancies, but not solid tumors. However, the discovery of *EML4–ALK* generated through inv (2) (p21p23) has changed this notion (9). This fusion gene encodes an oncogenic EML4–ALK fusion-type tyrosine kinase. Wild-type ALK is thought to undergo transient homodimerization in response to binding to its specific ligands, resulting in its activation. On the other hand, EML4–ALK is constitutively oligomerized via the coiled-coil domain within EML4, leading to persistent mitogenic signals that eventually lead to malignant transformation (9).

Brenner and Hall (12) described the risk of second malignancies associated with CT. Indeed, Mathews et al. (13) identified CT scans as a risk factor for second malignancies in a large cohort trial. However, the risk of lung cancer was not increased in their report, and the present patient received only one or two CT scans during his leukemia treatment and never underwent CT prior to that. Thus, we consider that the effect of irradiation was very limited in this secondary lung adenocarcinoma.

The occurrence of secondary *EML4–ALK*-positive lung cancer in the present case could have been the result of the patient's initial treatment for childhood ALL. Considering this case, known or unknown oncogenic recurrent chromosome translocations or DNA rearrangement might be present in other secondary epithelial malignancies, which should be carefully investigated by physicians who treat patients with all types of secondary malignancies.

Conflict of interest statement

The authors confirm that this report has not been published or presented elsewhere, either in whole or in part, and that it is not under consideration by another journal. All authors have approved the content and agree with its submission. No financial support was received for this publication, and none of the authors has any conflicts of interest to declare.

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ORIGINAL ARTICLE

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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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 five 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.

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Keywords: myeloperoxidase; acute myeloid leukemia; DNA methylation; DNA methyltransferase 3 beta

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 and World Health Organization 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 have important roles in

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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 *et al.*[24] 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. 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 five 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.

MATERIALS AND METHODS

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 two categories: high positivity ($>50\%$) of MPO enzymatic activity (MPOa-H) and low positivity ($\leqslant 50\%$) of MPO enzymatic activity (MPOa-L).

Culture of cell lines treated with DNMT-is

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; and 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 eight 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 Supplementary Experimental Procedures.

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 two healthy volunteers were purchased from Lonza Walkersville Inc. (Walkersville, MD, USA). The purity of CD34-positive cells assessed by flow cytometry was $>95\%$ after selection.

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).

Quantitative reverse transcriptase-PCR (QRT-PCR) for *MPO* and *DNMT3B* genes

MPO, DNMT 3 beta (*DNMT3B*) and Abelson tyrosine-protein kinase 1 (*ABL1*) transcription levels were quantitated using a QRT-PCR assay and shown as MPO/ABL1 and DNMT3B/ABL1 ratios. Detailed conditions, including primer sequences, are shown in the Supplementary Experimental Procedures.

DNA methylation microarray by Illumina infinium assay

This assay was performed as described previously.[25] Briefly, 4 μl of bisulfite-converted DNA (150 ng) 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 no.15019519A). 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 ($\beta =$ intensity of the Methylated allele (M)/intensity of the Unmethylated allele (U) + intensity of the Methylated allele (M) + 100).

Statistical methods

The results of the *in vitro* experiments are presented as the mean ± s.d. 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 four groups: MPOa-H AML group (a high percentage ($>50\%$) of MPO-positive myeloblasts in bone marrow smear), MPOa-L AML group (a low percentage ($\leqslant 50\%$) 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.

RESULTS

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) (Supplementary 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 $<25\%$, whereas that in all MPOa-L cell lines, except for one (K562), was above 60%, which was significant ($P=0.0038$, Supplementary 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 (Supplementary 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 (Supplementary 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.

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

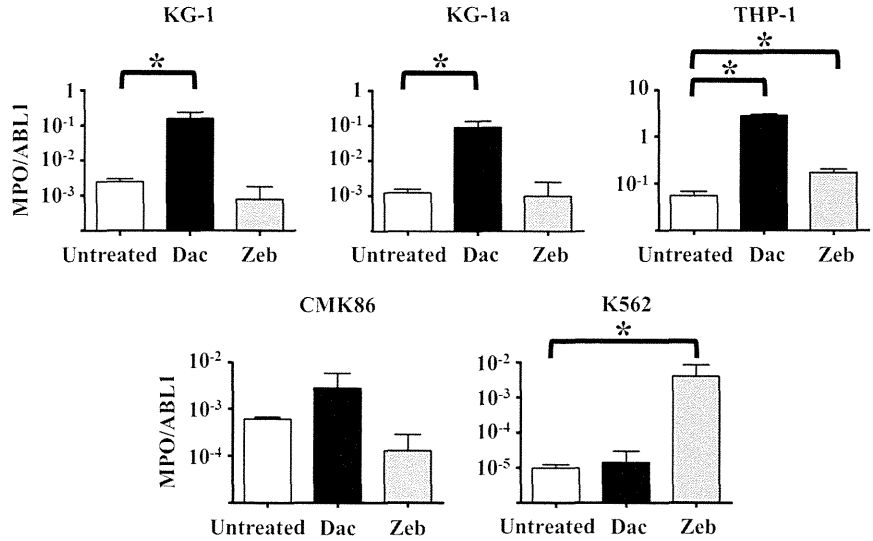

Figure 1. Expression of MPO mRNA in MPOa-L AML cell lines after the DNMT-i treatment. The expression of MPO mRNA in each cell line cultured with or without DNMT-i was analyzed by quantitative reverse transcriptase PCR (QRT-PCR). Results are expressed as the mean and s.d. of three independent studies (*$P<0.05$).

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 five AML patients (Unique patient number (UPN)-3, -12, -13, -14 and -15), an *NPM1* mutation in three AML patients (UPN-12, -13 and -15), *CEBPA* single mutation in one (UPN-4) and *CEBPA* double mutation in three AML patients (UPN-6, -7 and -9). The MPO positivity of blasts measured by cytochemistry and the amount of MPO mRNA are 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 three 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.

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 (that is, 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 (that is, both the enzymatic activity of the MPO protein and *MPO* gene expression levels were low), three Ph + ALL patients and two 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, whereas hypermethylation was noted in the same region in samples from the MPOa-L AML group (Supplementary 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.

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

Based on the 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 >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, 3433 probe sets were excluded from further analysis because of the lack of their gene symbol. The remaining probe sets covered 2658 and 3721 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 three other groups (MPOa-L AML, Ph + ALL and healthy donor groups),

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

UPNo.	*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: ALL, acute lymphoid leukemia; AML, acute myeloid leukemia; F, female; FAB, French–American–British classifications; M, male; NC, normal karyotype; UPN, unique patient number; WT, wild type. 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 DAB method. The MPO positivity of CD34-positive cells was also evaluated in cytospin slides.

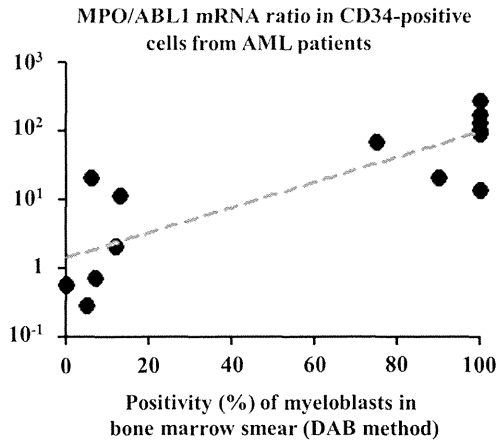

Figure 2. The significant relationship between the percentage of MPO-positive myeloblasts in bone marrow smears and the expression level of MPO mRNA in CD34-positive AML cells. The percentage of MPO-positive myeloblasts in bone marrow smears (estimated using the DAB method) showed a significant relationship with the amount of MPO mRNA (by QRT-PCR) in CD34-positive AML cells ($P<0.001$).

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 eight genes) clearly displayed hypomethylation at their CpG sites in the MPOa-H group, while those of 25 genes were hypermethylated relative to the three 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 (Supplementary 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.

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 (Supplementary 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).

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,

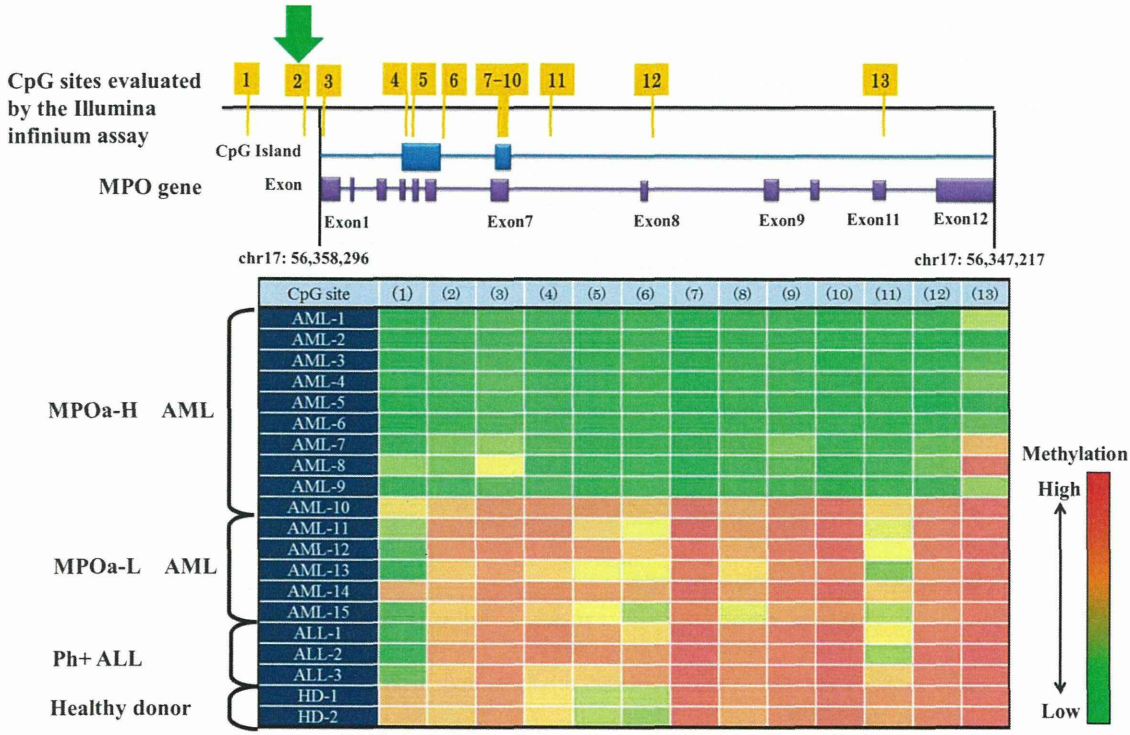

Figure 3. DNA methylation status of the *MPO* gene promoter and its gene body in CD34-positive AML cells. Quantification of DNA methylation in 13 CpG sites was performed using the Illumina infinium assay (color-coded as indicated by the scale at the left). The positions of the CpG sites (yellow square), 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).

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 *et al.*[23] reported that azacitidine induced *MPO* gene transcription in AML cell lines and AML blasts from patients. 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]

As 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 (Figures 4b, 5a and b). 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 *et al.*[47] reported that the overexpression of DNMT3B was an independent poor prognostic factor in AML patients. 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, isocitrate dehydrogenase 1

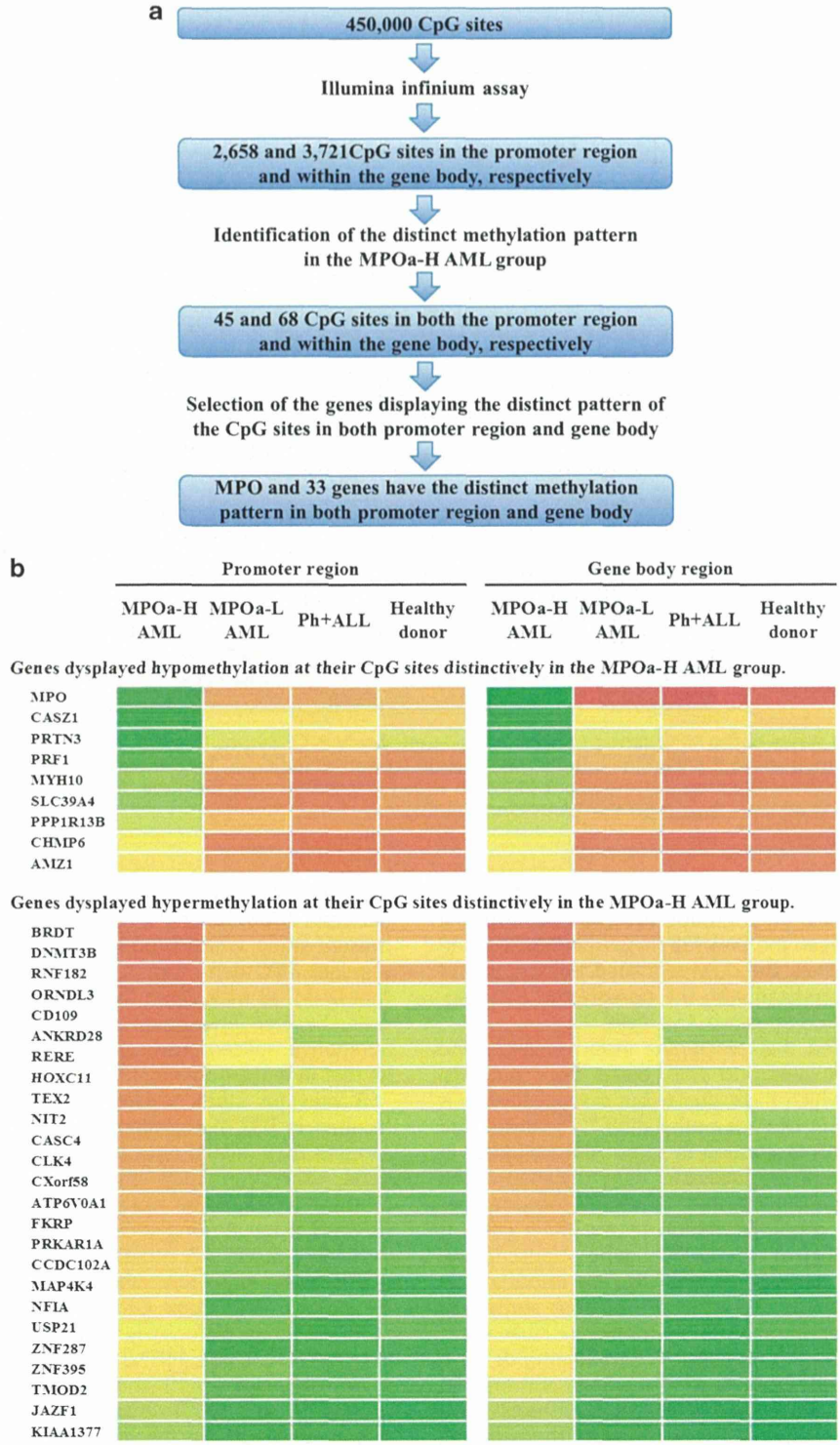

Figure 4. (**a**) Outline of the steps used to build the DNA methylation classifier. (**b**) Heatmap of the aberrant DNA methylation signature in CD34-positive cells obtained from MPOa-H AML. Data of MPOa-H AML patients were compared with those from MPOa-L AML and Ph + ALL patients and healthy donors. The gene symbols are indicated at the left.

(IDH1), IDH2, additional sex combs-like 1 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

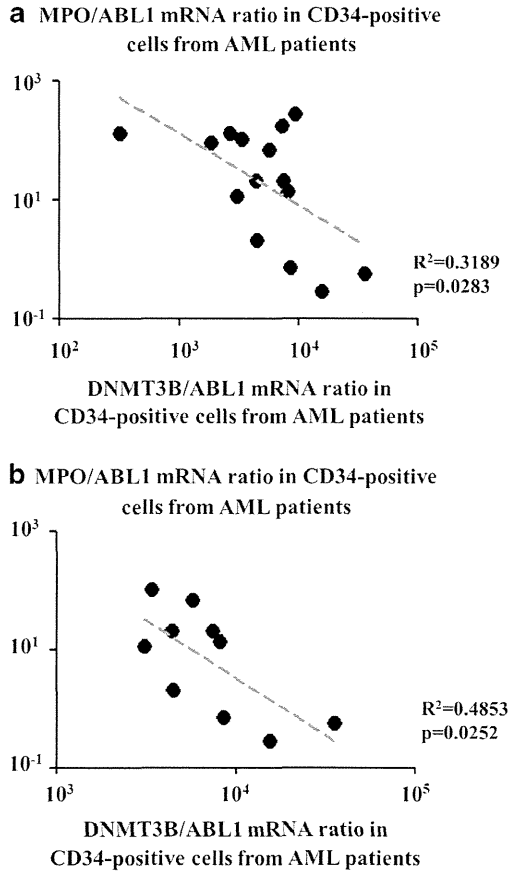

Figure 5. (**a**) Relationship between *MPO* and *DNMT3B* gene expression. A significant inverse relationship was observed between the amounts of MPO mRNA and DNMT3B mRNA in CD34-positive cells obtained from AML patients. Analysis for the relative expression level of DNMT3B mRNA was performed using QRT-PCR. (**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.

needed on the role of DNMT3B in AML and the use of DNMT-i for refractory AML.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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AUTHOR CONTRIBUTIONS

HI and Y Miyazaki conceived and designed the study; HI, DI, WYF, SS, KA, YS, DS, KT, HH, YI, JT, HT, SY, TF, TH, Y Moriuchi, KY and Y Miyazaki collected and analyzed the samples and data; HI 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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