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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*

H Itonaga[1,2], D Imanishi[1], Y-F Wong[3], S Sato[2], K Ando[1], Y Sawayama[1], D Sasaki[4], K Tsuruda[4], H Hasegawa[4], Y Imaizumi[1], J Taguchi[1], H Tsushima[2], S Yoshida[5], T Fukushima[6], T Hata[1], Y Moriuchi[2], K Yanagihara[4] and Y Miyazaki[1]

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

[1]Department of Hematology, Atomic Bomb Disease and Hibakusya Medicine Unit, Atomic Bomb Disease Institute, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan; [2]Department of Hematology, Sasebo City General Hospital, Sasebo, Japan; [3]Laboratory for Stem Cell Biology, RIKEN Center for Development Biology, Kobe, Japan; [4]Department of Laboratory Medicine, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan; [5]Department of Internal Medicine, National Hospital Organization Nagasaki Medical Center, Ohmura, Japan and [6]School of Health Sciences, University of the Ryukyus, Nishihara, Japan. Correspondence: Dr D Imanishi, Department of Hematology, Atomic Bomb Disease and Hibakusya Medicine Unit, Atomic Bomb Disease Institute, Nagasaki University Graduate School of Biomedical Sciences, 1-12-4 Sakamoto, Nagasaki 852-8523, Japan.
E-mail: idaisuke@nagasaki-u.ac.jp
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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

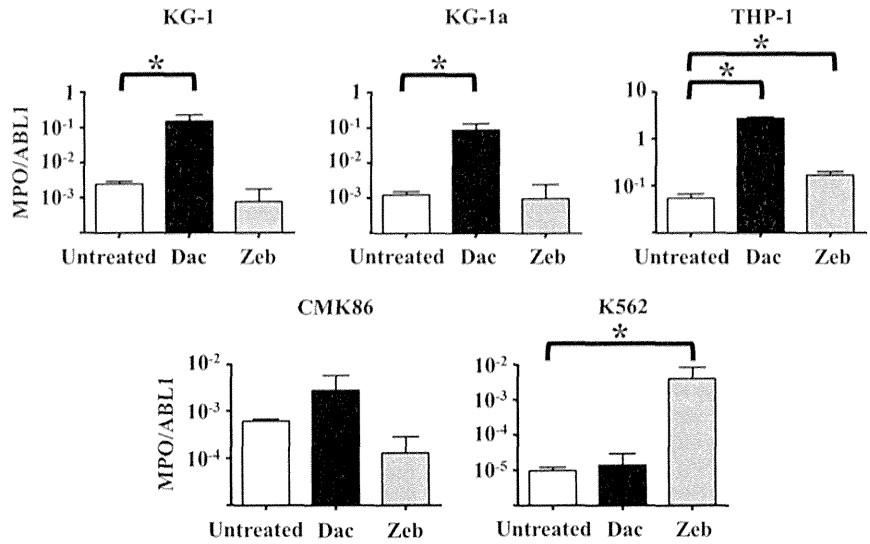


**Figure 1.** Expression of MPO mRNA in MPOa-L AML cell lines after the DNMT-i treatment. The expression of MPO mRNA in each cell line cultured with or without DNMT-i was analyzed by quantitative reverse transcriptase PCR (QRT-PCR). Results are expressed as the mean and s.d. of three independent studies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

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.

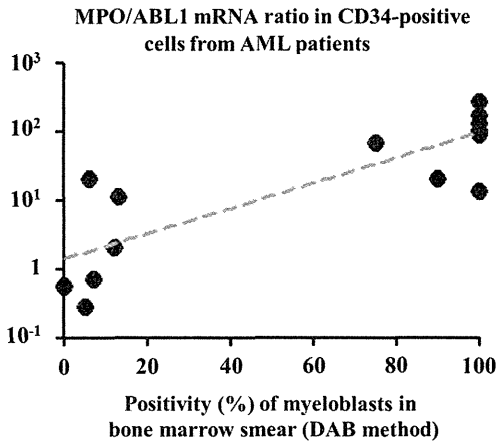


**Figure 2.** The significant relationship between the percentage of MPO-positive myeloblasts in bone marrow smears and the expression level of MPO mRNA in CD34-positive AML cells. The percentage of MPO-positive myeloblasts in bone marrow smears (estimated using the DAB method) showed a significant relationship 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,

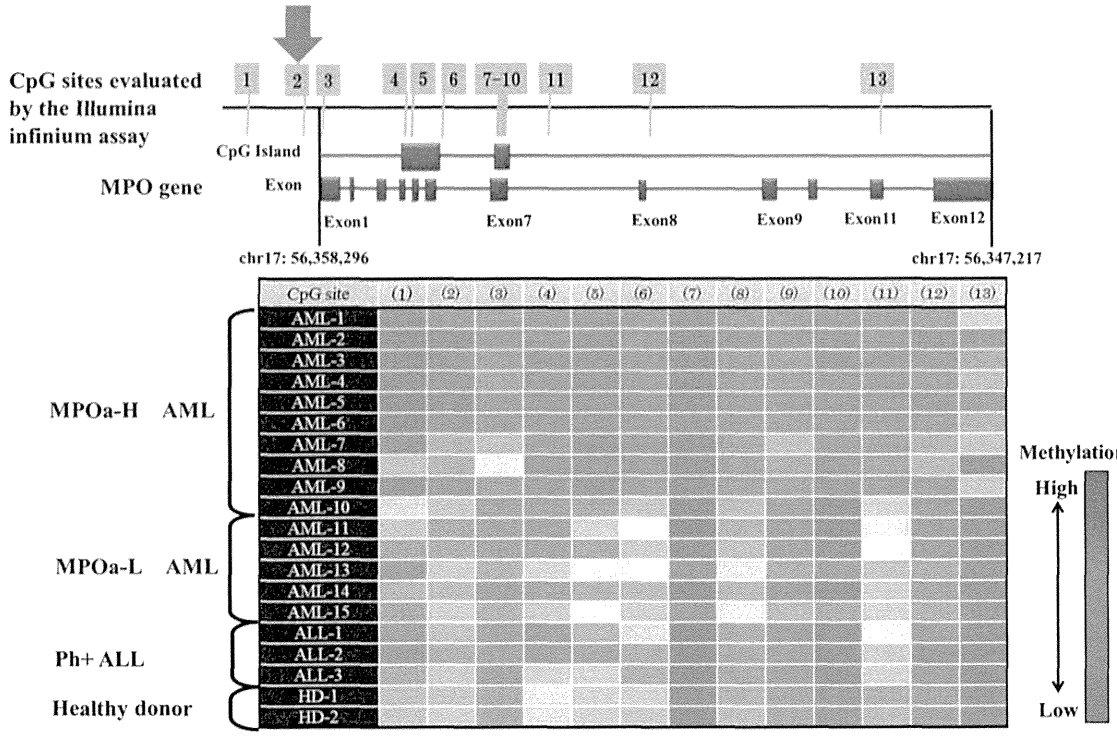


**Figure 3.** DNA methylation status of the *MPO* gene promoter and its gene body in CD34-positive AML cells. Quantification of DNA methylation in 13 CpG sites was performed using the Illumina infinium assay (color-coded as indicated by the scale at the left). The positions of the CpG sites (yellow square),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

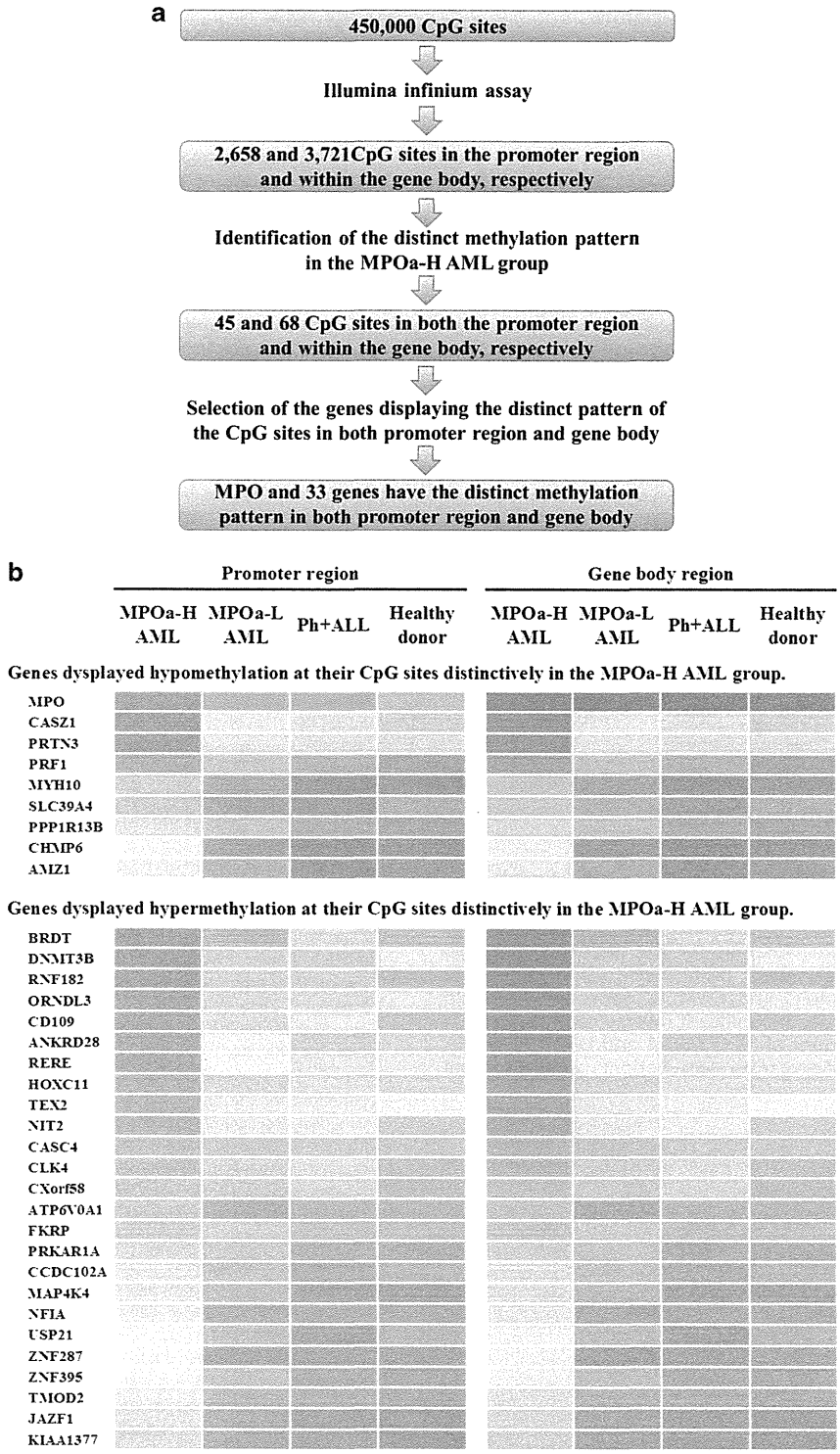


**Figure 4.** (**a**) Outline of the steps used to build the DNA methylation classifier. (**b**) Heatmap of the aberrant DNA methylation signature in CD34-positive cells obtained from MPOa-H AML. Data of MPOa-H AML patients were compared with those from MPOa-L AML and Ph + ALL patients and healthy donors.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

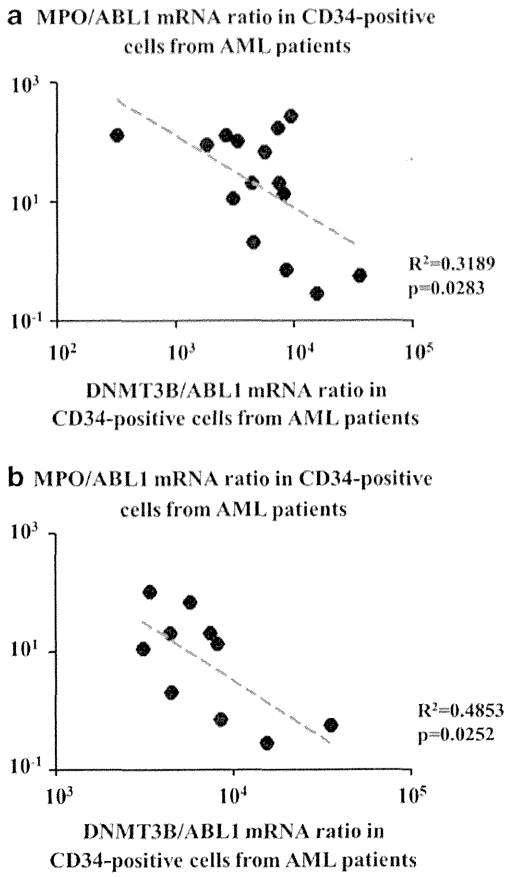


**Figure 5.** (**a**) Relationship between *MPO* and *DNMT3B* gene expression. A significant inverse relationship was observed between the amounts of MPO mRNA and DNMT3B mRNA in CD34-positive cells obtained from AML patients. Analysis for the relative expression level of DNMT3B mRNA was performed using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

# Normal karyotype acute myeloid leukemia with the CD7+ CD15+ CD34+ HLA-DR + immunophenotype is a clinically distinct entity with a favorable outcome

**Noriyoshi Iriyama · Norio Asou · Yasushi Miyazaki · Shunichiro Yamaguchi · Shinya Sato · Toru Sakura · Tomoya Maeda · Hiroshi Handa · Masatomo Takahashi · Shigeki Ohtake · Yoshihiro Hatta · Hisashi Sakamaki · Sumihisa Honda · Tomohiko Taki · Masafumi Taniwaki · Shuichi Miyawaki · Kazunori Ohnishi · Yukio Kobayashi · Tomoki Naoe**

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**Abstract** Recently, the presence of *CEBPA* mutation was identified as an important prognostic factor for normal karyotype (NK) acute myeloid leukemia (AML). Because AML with *CEBPA* mutation is closely associated with CD7, CD15, CD34, and HLA-DR expression, we investigated the prognostic implications of CD7+ CD15+ CD34+ HLA-DR + immunophenotype in NK-AML. We analyzed the immunophenotype of 329 patients with NK-AML from the Japan Adult Leukemia Study Group (JALSG) AML97 population. NK-AML with the CD7+ CD15+ CD34+ HLA-DR + immunophenotype was classified as the CEBPA type, and NK-AML that did not meet this criterion was considered as

N. Iriyama (✉) · Y. Hatta
Department of Hematology and Rheumatology, Nihon University School of Medicine, 30-1 Oyaguchi Kamicho, Itabashi-ku, Tokyo 173-8610, Japan
e-mail: iriyama.noriyoshi@nihon-u.ac.jp

N. Asou · T. Maeda
Department of Hematology, Saitama Medical University International Medical Center, Saitama, Japan

N. Asou · S. Yamaguchi
Department of Hematology, Kumamoto University School of Medicine, Kumamoto, Japan

Y. Miyazaki
Department of Hematology, Atomic Bomb Disease Institute, Nagasaki University, Nagasaki, Japan

S. Sato
Department of Hematology, Sasebo City General Hospital, Nagasaki, Japan

T. Sakura
Department of Hematology, Saiseikai Maebashi Hospital, Gunma, Japan

H. Handa
Department of Medicine and Clinical Sciences, Gunma University Graduate School of Medicine, Maebashi, Gunma, Japan

M. Takahashi
Division of Hematology and Oncology, St. Marianna University School of Medicine, Kawasaki, Japan

S. Ohtake
Clinical Laboratory Science, Division of Health Sciences, Kanazawa University Graduate School of Medical Sciences, Kanazawa, Japan

H. Sakamaki
Department of Hematology, Tokyo Metropolitan Cancer and Infectious Diseases Center Komagome Hospital, Tokyo, Japan

S. Honda
Department of Public Health, Nagasaki University Graduate School of Biomedical Sciences, Nagasaki, Japan

T. Taki
Department of Molecular Diagnostics and Therapeutics, Kyoto Prefectural University of Medicine, Kyoto, Japan

M. Taniwaki
Department of Hematology and Oncology, Kyoto Prefectural University of Medicine Graduate School of Medical Science, Kyoto, Japan

S. Miyawaki
Division of Hematology, Tokyo Metropolitan Ohtsuka Hospital, Tokyo, Japan

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the non-CEBPA type. The influence of the CEBPA status on event-free survival (EFS) and overall survival (OS) was assessed using log-rank test and a multivariate Cox proportional hazard regression model. Furthermore, the surface antigen expression profile in AML according to the *CEBPA* mutation status (monoallelic or biallelic) was also investigated. Of the 329 NK-AML patients that were studied, 39 and 243 were classified as having CEBPA and non-CEBPA type NK-AML, respectively. Patients with CEBPA type NK-AML had significantly better EFS and OS than those with non-CEBPA type NK-AML. Multivariate analysis showed that the CEBPA type and white blood cell (WBC) counts of $>20 \times 10^9$/L were independent prognostic factors for EFS and OS. Moreover, NK-AML with the biallelic *CEBPA* mutation was more closely associated with CD34 positivity than that with the monoallelic *CEBPA* mutation. NK-AML with the CD7+ CD15+ CD34+ HLA-DR + immunophenotype is a clinically discrete entity, and this may have a possible role in risk stratification.

**Keywords** Normal karyotype acute myeloid leukemia · CD7 · CD15 · CD34 · HLA-DR · CEBPA · Prognostic factor

## Introduction

In recent years, immunophenotyping of hematologic neoplasms has become standard practice to establish a diagnosis and define the origin of the malignant cell lineage. Patients with acute myeloid leukemia (AML) often show aberrant cellular antigen expression as well as chromosomal abnormalities. The clinical significance of surface antigen expression has been studied for more than 20 years, but thus far, it has yielded inconsistent results [1]. Nevertheless, if the evaluation of antigen expression is limited to a subtype of AML, we would be more likely to find a significant relationship between surface antigen expression and prognosis. For example, the significance of CD56 expression as an adverse prognostic factor in both acute promyelocytic leukemia (APL) and AML with t(8;21) is widely accepted [2, 3].

Normal karyotype (NK)-AML is the most common subtype of AML, accounting for 40–50 % of cases [4-6]. Patients with this subtype are considered to have an intermediate risk, and upfront hematopoietic stem cell transplantation (HSCT) is commonly recommended [7-9]. However, even though treatment strategies seem promising, the prognosis of NK-AML is variable when molecular evaluation of the *FLT3*, *NPM1*, and *CEBPA* mutations is taken into account [10-14]. Schlenk et al. [10] reported that NK-AML patients with the *CEBPA* mutation or *NPM1* mutation, but without the *FLT3* mutation, had a favorable prognosis and that upfront HSCT in these patients did not contribute to the overall survival (OS). Although the detection of subgroups is necessary for decisions on the most appropriate treatment strategy, routine molecular diagnoses are often difficult in clinical practice.

AML with the *CEBPA* mutation has a homologous surface antigen expression that is closely associated with CD7, CD15, CD34, and HLA-DR positivity [15, 16]. We speculated that immunophenotyping for CD7, CD15, CD34, and HLA-DR in patients with NK-AML could identify a distinct subtype of AML that clinically mimics AML with the *CEBPA* mutation. In the Japan Adult Leukemia Study Group (JALSG) AML97 study, almost 42 % of the patients with AML were diagnosed with NK-AML. For this study, results of surface antigen expression were obtained at the time of enrollment. Further, we used data from the JALSG AML97 study to investigate the clinical significance of these surface antigens for the prognosis of patients with NK-AML.

## Patients and methods

### Patients

We conducted a retrospective review of patient data from the multicenter JALSG AML97 study. Detailed information of this study and its results has previously been reported [17, 18]. Briefly, between December 1997 and July 2001, patients aged 15–64 years, with newly diagnosed de novo AML, excluding those with APL, were consecutively enrolled to the JALSG AML97 study. In total, 789 of the 809 AML patients were eligible for the study, and informed consent was obtained from all patients or their guardians before enrollment. The study protocol was approved by the research ethics boards of all participating institutions, and the study was conducted in accordance with the Declaration of Helsinki.

### Cytogenetic studies

The results from the cytogenetic studies, which were performed at each of the institutions, were reported to the JALSG Statistical Center. Routinely, 20 metaphases were counted for each patient and analyzed according to the recommendations of the International System for Human Cytogenetic Nomenclature.

K. Ohnishi
Oncology Center, Hamamatsu University School of Medicine, Shizuoka, Japan

Y. Kobayashi
Hematology Division, National Cancer Center Hospital, Tokyo, Japan

T. Naoe
National Hospital Organization Nagoya Medical Center, Nagoya, Japan

### Flow cytometry

Immunophenotyping was performed at each institution, primarily on freshly collected bone marrow or peripheral blood samples that were collected at the time of diagnosis. Leukemic cell analysis was performed at local or reference laboratories by standard immunofluorescence methods using monoclonal antibodies directed against the CD2, CD3, CD4, CD5, CD7, CD8, CD11b, CD13, CD15, CD19, CD33, CD34, CD41a, CD56, and HLA-DR surface antigens. Samples were considered positive if at least 20 % of blasts expressed the antigen.

### Treatment regimen used in the JALSG AML97 study

Induction therapy consisted of Ara-C at a dose of 100 mg/m$^2$ per day as a continuous infusion on days 1–7 and idarubicin (IDR) at a dose of 12 mg/m$^2$ per day as a 30-min infusion on days 1–3. Patients who did not achieve remission after the first induction cycle were given the same therapy again. Patients who obtained complete remission (CR) within two courses of induction therapy were randomly assigned to a group that received either four courses of standard dose consolidation therapy without maintenance (arm A) or three courses of standard dose consolidation along with six courses of maintenance therapy (arm B). In the JALSG AML97 study, the 5-year overall survival rate and the 5-year disease-free survival (DFS) rate between the arms were not statistical different [17].

### Surface antigen expression profile according to the CEBPA mutant pattern

We also investigated the surface antigen expression profiles according to *CEBPA* mutant pattern in 318 AML patients based on the data records of AML patients enrolled at the Kumamoto and Nagasaki Universities. High molecular weight genomic DNA was extracted from the bone marrow or peripheral blood samples after Ficoll separation of mononuclear cells. Mutations of the *CEBPA* gene was detected by genomic DNA PCR, and direct sequencing was performed at each institution, as described previously [19, 20].

### Statistical analysis

OS for all patients was defined as the period from the date of diagnosis to the date of death. Event-free survival (EFS) was defined as the period from the date of diagnosis to the date of the first recurrence after CR or any cause of death. All patients who underwent HSCT were censored from the EFS analysis on the date of HSCT treatment. The Kaplan-Meier method was used to estimate the EFS and OS. The log-rank test was used to compare the EFS or OS of the two groups. Factors that could potentially affect clinical outcome, including age, sex, WBC count, performance status at diagnosis, and the expression of each surface antigen were analyzed by the multivariate Cox proportional hazard regression model. Fisher's exact test and Student's *t* test were used to compare factor differences between the two groups. Statistical analysis was performed with the JMP software version 8.0.1 (SAS Institute Inc., Cray, NC, USA).

## Results

### Definition of the CEBPA type

For this study, CEBPA type NK-AML was defined as NK-AML that showed the CD7+ CD15+ CD34+ HLA-DR + immunophenotype because these antigens are commonly expressed in AML with the *CEBPA* mutation [15, 16]. Non-CEBPA type NK-AML was defined as NK-AML that did not have the CD7+ CD15+ CD34+ HLA-DR + immunophenotype.

In total, 329 patients were diagnosed with NK-AML. The expression of CD7, CD15, CD34, and HLA-DR was examined in 303, 201, 306, and 302 patients, respectively. Of the

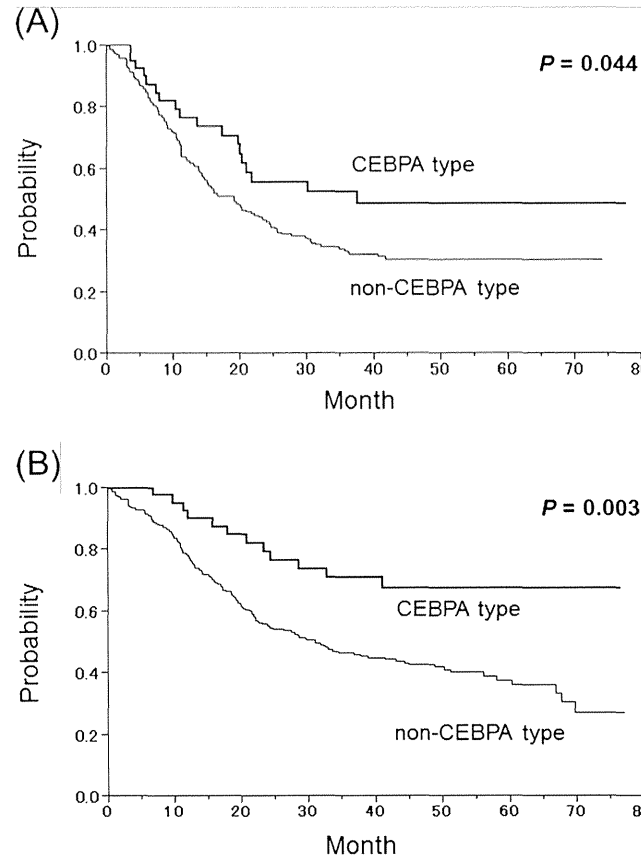


**Fig. 1** Kaplan-Meier curves show event-free survival (EFS) and overall survival (OS) according to the CEBPA status. EFS and OS for each group are shown in **a** and **b**, respectively. Log-rank test revealed the 5-year EFS to be 48.5 and 30.5 % for patients with CEBPA and non-CEBPA type NK-AML, respectively, which was significantly different ($P$=0.044). The 5-year OS of patients with CEBPA and non-CEBPA type NK-AML was also significant (67.4 and 35.7 %, respectively; $P$=0.0038)

**Table 1** Analysis of prognostic factors for event-free survival in the NK-AML population ($n$=329)

| Factors | Number (positive/negative) | Univariate analysis | | Multivariate analysis | |
|---|---|---|---|---|---|
| | | HR (95 % CI) | $P$ value | HR (95 % CI) | $P$ value |
| Age > 50 years | 131/198 | 1.04 (0.77–1.39) | 0.812 | | |
| Female sex | 141/188 | 0.81 (0.60–1.09) | 0.168 | | |
| WBC count of $>20\times10^9$/L | 165/164 | 1.74 (1.30–2.34) | 0.0002 | 1.56 (1.14–2.14) | 0.0052 |
| Performance status ≥2 | 29/300 | 1.21 (0.72–1.91) | 0.461 | | |
| CD7 | 108/195 | 0.96 (0.70–1.31) | 0.808 | | |
| CD15 | 113/88 | 0.77 (0.52–1.13) | 0.174 | | |
| CD34 | 166/140 | 1.26 (0.94–1.71) | 0.127 | | |
| HLA-DR | 255/47 | 1.16 (0.77–1.82) | 0.483 | | |
| CEBPA type | 39/243 | 0.61 (0.36–0.96) | 0.034 | 0.59 (0.35–0.94) | 0.026 |

329 NK-AML patients, 39 were classified as having the CEBPA type and 243 were classified as having the non-CEBPA type NK-AML. We excluded 47 patients whose of immunophenotype could not be determined.

## EFS and OS

The 5-year EFS rate for patients with CEBPA type NK-AML was 48.5 %, which was significantly higher than the 30.5 % for patients with non-CEBPA type NK-AML ($P$=0.044, Fig. 1a). Furthermore, the 5-year OS rate for patients with CEBPA type NK-AML was also significantly higher than that for patients with non-CEBPA type NK-AML (67.4 vs 35.7 %, $P$=0.0038, Fig. 1b).

Univariate analysis showed that the outcome of patients with increased WBC counts at diagnosis was significantly worse (Tables 1 and 2), in agreement with previous reports [1]. Furthermore, CEBPA type was also a significant factor for better EFS and OS (Tables 1 and 2). Multivariate analysis showed CEBPA type and increased WBC counts to be independent prognostic factors (Tables 1 and 2). Other factors such as age, performance status, sex, or the expression of each of the single surface antigens did not affect the EFS and OS rates.

Our study included 12 patients with CEPBA type NK-AML and 77 patients with non-CEBPA type NK-AML who received HSCT. There was no significant difference among patients who received HSCT in these two groups (Table 3). The 2-year OS after HSCT in these groups were 61 and 41 %, respectively, which did not reach significance ($P$=0.467).

## Clinical profiles in CEBPA type

The CEBPA type was identified as an independent prognostic factor for EFS and OS. Therefore, we analyzed the characteristics of CEBPA type (Table 3). Sex, WBC count, or performance status was not associated with CEBPA or non-CEBPA type NK-AML. In contrast, compared with non-CEBPA type NK-AML, CEBPA type NK-AML was associated with younger age, higher myeloperoxidase (MPO)-positive rates, frequent presentation with Auer rods, and a French-American-British (FAB) classification of M1 or M2.

**Table 2** Analysis of prognostic factors for overall survival in the NK-AML population ($n$=329)

| Factors | Number (positive/negative) | Univariate analysis | | Multivariate analysis | |
|---|---|---|---|---|---|
| | | HR (95 % CI) | $P$ value | HR (95 % CI) | $P$ value |
| Age > 50 years | 131/198 | 1.19 (0.89–1.60) | 0.240 | | |
| Female sex | 141/188 | 0.80 (0.59–1.08) | 0.144 | | |
| WBC count of $>20\times10^9$/L | 165/164 | 1.51 (1.13–2.03) | 0.0059 | 1.44 (1.05–1.97) | 0.023 |
| Performance status ≥2 | 29/300 | 1.23 (0.72–1.96) | 0.437 | | |
| CD7 | 108/195 | 0.79 (0.57–1.09) | 0.150 | | |
| CD15 | 113/88 | 0.72 (0.49–1.07) | 0.101 | | |
| CD34 | 166/140 | 1.10 (0.81–1.50) | 0.526 | | |
| HLA-DR | 255/47 | 0.95 (0.64–1.47) | 0.807 | | |
| CEBPA type | 39/243 | 0.41 (0.22–0.71) | 0.0008 | 0.40 (0.21–0.69) | 0.0005 |

**Table 3** Correlations of the clinical profiles and treatments in patients with CEBPA and non-CEBPA type NK-AML

| Factors | CEBPA type ($n$=39) | Non-CEBPA type ($n$=243) | $P$ value |
|---|---|---|---|
| Clinical profile | | | |
| Age (year), median (range) | 38 (16–64) | 48 (15–64) | 0.018 |
| Sex (male/female) | 27:12 | 138:105 | 0.164 |
| WBC count ($\times 10^9$/L), median (range) | 21.8 (3.0–449.5) | 19.1 (0.6–709.0) | 0.301 |
| Auer rod: positive/negative | 31/7 | 101/139 | <0.0001 |
| FAB M1 or M2/others | 37/2 | 122/121 | <0.0001 |
| MPO positivity of >50 %/≤50 % | 30/6 | 95/105 | <0.0001 |
| Performance status ≥2/0–1 | 4/35 | 19/224 | 0.538 |
| HSCT | 12 | 77 | 1.000 |
| CR1 | 4 | 33 | 0.798 |
| CR2 | 5 | 14 | 0.157 |
| Others | 3 | 30 | 0.592 |

*MPO* myeloperoxidase, *FAB* French-American-British classification, *HSCT* hematopoietic stem cell transplantation, *CR* complete remission

Surface antigen profiles according to *CEBPA* mutant pattern

We investigated the expression of surface antigens according to monoallelic or biallelic *CEBPA* mutation in patients with AML. Of the 318 AML patients, 41 were diagnosed with the *CEBPA* mutation, which included 29 with biallelic and 12 with monoallelic mutations. The presence of the *CEBPA* mutation was common in intermediate risk AML, including NK-AML. In contrast, the mutation was uncommon in core-binding factor (CBF)-AML and adverse risk AML.

To investigate the association between surface antigen expression and *CEBPA* mutant pattern in NK-AML patients, the expressions of CD7, CD34, and HLA-DR were examined (Table 4). There was a significantly higher frequency of CD34 expression in AML patients with biallelic *CEBPA* mutation than in those with monoallelic mutation.

## Discussion

The results of this study show that the CD7+ CD15+ CD34+ HLA-DR + immunophenotype is a significant predictor of OS in patients with NK-AML. We were able to analyze data from a well-designed, uniform, prospective study. In addition, we observed that CEBPA type NK-AML was a discrete clinical entity, which is closely associated with high MPO positivity rates, Auer rod positivity, FAB classification of M1 or M2, and a younger age. These characteristics are very similar to the characteristics of AML with *CEBPA* mutation as previously reported: AML with the *CEBPA* mutation also had high MPO rates [19], FAB classification of M1 or M2 [21], and better OS [10, 11]. In addition, the prognosis of CEBPA type NK-AML was almost similar to that in the favorable risk group of AML as indicated by the JALSG scoring system [17] and that in AML patients with the *CEBPA* mutation [10, 11], as previously reported. Moreover, of 282 patients with NK-AML, 14 % had CEBPA type NK-AML, which is similar to that observed in previous reports (i.e., 10–18 % of patients with NK-AML had the *CEBPA* mutation) [22].

**Table 4** Surface antigen expression in AML patients according to monoallelic or biallelic *CEBPA* mutation

| Factors | Monoallelic *CEBPA* mutation Positive/negative (%) | Biallelic *CEBPA* mutation Positive/negative (%) | $P$ value |
|---|---|---|---|
| CD7 | 7/3 (70) | 23/4 (85) | 0.360 |
| CD34 | 5/5 (50) | 25/2 (93) | 0.009 |
| HLA-DR | 11/0 (100) | 23/2 (92) | 1.000 |

It is also known that the expressions of CD7, CD34, and HLA-DR are associated with poor clinical outcomes in AML [1]. In contrast to previous reports, our study, which was limited to NK-AML patients, demonstrated that the expression of single surface antigens including CD7, CD15, CD34, and HLA-DR did not have prognostic significance. Moreover, the CEBPA immunophenotype demonstrated a favorable OS even though the population positively expressed CD7, CD34, and HLA-DR, which have previously been considered poor prognostic factors.

It was previously reported that CD7 expression is associated with the *FLT3* mutation [23], *CEBPA* mutation [15, 16], and adverse risk cytogenetics [2, 24]. Furthermore, CD34 positivity is frequently observed in patients with adverse risk cytogenetics and t(8;21) AML [2], and it is negatively associated with *NPM1* mutations [25]. Another surface antigen, the B-cell marker CD19, which is observed in t(8;21) AML and that is associated with negativity for *KIT* mutation, favorably affects CR in AML patients with t(8;21) [2, 26]. Taken together, these results represent the difficulty in analyzing the heterogeneous population of AML as a total group because the impact of chromosomal and/or molecular abnormalities on prognosis makes it difficult to conclusively interpret the significance of surface antigen expression.

On the other hand, the population, which we detected with this combination of surface antigens, is evidently a distinct subtype of NK-AML with a discrete clinical profile (Table 3). Our findings show that specific subtypes of AML such as NK-AML with the *CEBPA* mutation potentially exist, and it suggests that the population of patients who have a favorable prognosis may well be identified through an analysis of the surface antigens that are expressed. Therefore, if the molecular evaluation is unavailable, an analysis of surface antigens may help in identifying patients with a favorable prognosis.

It is known that biallelic *CEBPA* mutations, but not monoallelic mutations, have a favorable prognosis [27]. Our study, which included all cases with the *CEBPA* mutation, showed that 93 % of cases with biallelic mutation were positive for CD34, while only 50 % of cases with monoallelic mutation were positive (Table 4), suggesting that CD34 is an important factor for distinguishing the mutant pattern. Because low activity of *CEBPA* is thought to be a critical factor for sustaining the immature character of AML cells [28], we hypothesized that a more potent inactivation of *CEBPA* by biallelic mutation results in the frequent incidence of CD34 positivity.

In this study, immunophenotypic analyses were not performed at a central facility. Another limitation of our study is the possible selection bias; not all the institutes performed cytometric analysis of all AML97 study antigens at the time of patient enrollment. This resulted in a reduction in sample size, because CEBPA status was not detected in 14 % of patients with NK-AML. JALSG has recently investigated whether molecular evaluations, including that of the *CEBPA* mutation, affect patient prognosis [29]. In the future, we hope to clarify the relationships among surface antigen expression, cytogenetics, molecular evaluation results, and clinical features of AML. In conclusion, we found that the CD7+ CD15+ CD34+ HLA-DR + immunophenotype has a potential role in risk stratification for patients with NK-AML.

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