Expression of myeloperoxidase and gene mutations in AML patients with normal karyotype: Double *CEBPA* mutations are associated with high percentage of MPO-positivity in leukemic blasts

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ABSTRACT

The percentage of myeloperoxidase (MPO)-positive blast cells is a simple and highly significant prognostic factor in AML patients. It has been reported that the high MPO group (MPO-H), in which >50% of blasts are MPO activity positive, is associated with favorable karyotypes, while the low MPO group (≤50% of blasts are MPO activity positive, MPO-L) is associated with adverse karyotypes. The MPO-H group shows better survival even when restricted to patients belonging to the intermediate chromosomal risk group or those with a normal karyotype. It has recently been shown that genotypes defined by the mutational status of *NPM1*, *FLT3*, and *CEBPA* are associated with treatment outcome in patients with cytogenetically normal AML. In this study, we aimed to evaluate the relationship between MPO positivity and gene mutations found in normal karyotypes. Sixty AML patients with normal karyotypes were included in this study. Blast cell MPO positivity was assessed in bone marrow smears stained for MPO. Associated genetic lesions (the *NPM1*, *FLT3*-ITD, and *CEBPA* mutations) were studied using nucleotide sequencing. Thirty-two patients were in the MPO-L group, and 28 patients in the MPO-H group. *FLT3*-ITD was found in 11 patients (18.3%), *NPM1* mutations were found in 19 patients (31.7%), and *CEBPA* mutations were found in 11 patients (18.3%). In patients with *CEBPA* mutations, the carrying two simultaneous mutations ($CEBPA^{double\text{-}mut}$) was associated with high MPO expression, while the mutant *NPM1* without *FLT3*-ITD genotype was not associated with MPO activity. Both higher MPO expression and the $CEBPA^{double\text{-}mut}$ genotype appeared to be associated with improved overall survival after intensive chemotherapy. Further studies are required to determine the importance of blast MPO activity as a prognostic factor, especially in *CEBPA* wild-type patients with a normal karyotype.

Keywords:
acute myeloid leukemia, normal karyotype, myeloperoxidase, *CEBPA* mutations

1. Introduction

The AML87, -89, and -92 studies conducted by Japan Adult Leukemia Study Group (JALSG) revealed that patient age, ECOG performance status, leukocyte count, FAB subclass, the number of induction courses required to achieve complete remission (CR), the presence of good prognostic chromosomal abnormalities (t(8;21) or inv(16)), and percentage of myeloperoxidase (MPO)-stained positive blast cells at diagnosis were significant risk factors for overall survival (OS) of patients with acute myeloid leukemia (AML) [1]. In more recent AML201 study, it was shown that significant unfavorable prognostic features for OS were adverse cytogenetic risk group [2], age of more than 50 years, WBC more than 20 x 10^9/L, FAB classification of either M0, M6, or M7, and MPO-positive blasts less than 50% [3]. These observations imply that the percentage of MPO-positive blast cells is one of the important prognostic markers along with cytogenetics and molecular genetic information.

MPO, a microbiocidal protein, is considered to be a golden marker for the diagnosis of AML in the French-American-British (FAB) and WHO classifications [4-5]. In our previous reports [6-8], AML patients with a high percentage of MPO positive blasts (>50% of blasts are MPO activity positive, MPO-H) had a significantly better complete remission (CR) rate, disease-free survival, and overall survival compared with the low MPO activity positive blast group (≤50% of blasts are MPO activity positive, MPO-L). Most patients with a favorable chromosomal risk profile were in the MPO-H group, and most of the patients with an adverse chromosomal risk profile were in the MPO-L group. The difference in OS between the low and high MPO groups was still observed in a cohort of patients with normal karyotypes, suggesting that MPO is highly expressed in the leukemic blasts of AML patients with a favorable prognosis. To fully understand this phenomenon, it would be important to analyze genetic factors associated with MPO expression, especially in patients with a normal karyotype.

In the WHO classification, mutations of *FLT3*, *NPM1* and *CEBPA* have been emphasized to have prognostic significance in AML patients with normal karyotype. The nucleophosmin 1 gene (*NPM1*) has been shown to be mutated in 45-64% of AML cases with a normal karyotype [9-10], and *NPM1* mutations are associated with a favorable prognosis in the absence of the internal tandem duplication (ITD) type of fms-related tyrosine kinase-3 gene (*FLT3*) mutation, a known adverse prognostic factor [11]. The CCAAT/enhancer binding protein-alpha gene (*CEBPA*) is another gene that has been shown to be mutated in AML patients with a normal karyotype [12-13]. Mutations in the *CEBPA* gene are found in 5% to 14% of all AML cases and are associated with a relatively favorable outcome, and hence, have gained interest as a prognostic marker [14]. Recently, it has been shown that most AML patients with *CEBPA* mutations carry 2 simultaneous mutations ($CEBPA^{double\text{-}mut}$) whereas single mutations ($CEBPA^{single\text{-}mut}$) are less common. In addition it was found that the $CEBPA^{double\text{-}mut}$ genotype is associated with a favorable overall and event-free survival [15-16]. It is still unclear why $CEBPA^{double\text{-}mut}$ AML patients have better outcomes than those with a single heterozygous mutation.

In this study, we retrospectively examined 60 de novo adult AML patients with normal karyotypes in order to obtain a better insight into the relationships between MPO positivity and other prognostic factors (*NPM1*, *FLT3*, and *CEBPA* mutations). In line with previous reports, both high MPO positivity in AML

blasts and the *CEBPA*$^{\text{double-mut}}$ genotype appeared to be associated with a favorable outcome, and it appeared that it was the *CEBPA*$^{\text{double-mut}}$ genotype that associated with high blast MPO activity.

2. Materials and methods

2.1 Patients and treatments

The study population included 60 patients with newly diagnosed de novo AML that had been treated at the Department of Internal Medicine, Nagasaki National Medical Center, between 1990 and 2010. All patients had normal karyotype AML. AML was diagnosed according to the FAB classification. Two members independently assessed the percentage of MPO-positive blast cells in MPO-stained bone marrow smears. The main biological and clinical features of the patients are shown in Table 1. Excluding the 25 patients who did not receive conventional induction chemotherapy, all patients were treated according to the Japan Adult Leukemia Study Group (JALSG) protocols (AML 89, 92, 95, 97, and 201 studies) [3, 17-19]. CR was determined as when blasts accounted for less than 5% of the cells in normocellular bone marrow with normal peripheral neutrophil and platelet counts. This study was approved by the Ethical Committees of the participating hospitals.

2.2 Analysis of the *FLT3*, *NPM1*, and *CEBPA* genes.

High molecular weight genomic DNA was extracted from bone marrow and peripheral blood samples after Ficoll separation of mononucleated cells (35 and 4 patients, respectively) using the QIAamp DNA Mini Kit (Qiagen, Hilden, Germany). In addition, we isolated genomic DNA from the BM smears of the AML patients (21 samples) using the QIAamp DNA blood Mini Kit (Qiagen, Hilden, Germany).

Mutations in the *FLT3*, *NPM1*, and *CEBPA* genes were detected by genomic DNA PCR and direct sequencing. Exons 14 and 15 and the intervening intron of the *FLT3* gene were amplified from DNA using the previously described primers FLT3-11F and FLT3-12R [20]. PCR for NPM1 exon 12 was performed with genomic DNA, the same reagent, and the published primer molecules NPM1-F and NPM1-R [21]. PCR for *CEBPA* was performed using 2 overlapping primer pairs: CEBPA-CT3F (5'-TGCCGGGTATAAAA-GCTGGG-3') and CT3R (5'-CTCGTTGCTGTTCTTGTCCA-3'), CEBPA-PP2F (5'-TGCCGGGT-ATAAAAGCTGGG-3') and PP2R (5'-CACGGTCTGGGCAAGCCTCGAGAT-3'). The PCR reactions were run in a final volume of 50 μL containing 10 ng DNA, 5×buffer, 0.2 mmol/L of each deoxynucleotide triphosphate, primers (0.3 μmol/L of each), nucleotides (0.2 mmol/L of each), and 1 U of KOD-Plus-Neo polymerase (TOYOBO, Osaka, Japan). The mixture was initially heated at 94°C for 2 minutes, before being subjected to 35 cycles of denaturation at 94°C for 10 seconds and annealing and extension at 68°C for 1 minute. The amplified products were cut out from a 1.2% agarose gel and purified with the MinElute Gel extraction kit (QIAGEN, Germany). To screen for mutations, the PCR products were sequenced in both directions with the following primers: FLT3-11F, FLT3-12R, NPM1-F, NPM-R, CEBPA-CT1F, CEBPA-1R,

CEBPA-PP2F, CEBPA-PP2R, CEBPA-2F (5'-GCTGGGCGGCATCTGCG-A-3'), and CEBPA-1R (5'-TGT-GCTGGAACAGGTCGGCCA-3') using a BigDye Terminator v3.1 Cycle Sequencing Kit and the ABI Prism 3100 xl Genetic Analyzer (Applied Biosystems, CA, USA). In the case of *NPM1* and *CEBPA* genes, when heterozygous data were identified by sequence screening, mutations were confirmed by cloning with the StrataClone Blunt PCR Cloning Kit (Stratagene, CA, USA) according to the manufacturer's recommendations. Four to Ten recombinant colonies were chosen and cultured in LB medium. Plasmid DNA was prepared using a QIAprep spin plasmid miniprep kit (Qiagen, Hilden, Germany), and both strands were sequenced using the T3 and T7 primers and the CEBPA-2F and CEBPA-1R primers.

2.3 Statistical methods

To evaluate the relationship between the frequency of mutations status and clinical characteristics, the following variables were included in the analysis: age, FAB classification, peripheral WBC count, MPO-positivity rate, JALSG score [1], and CR achievement. A comparison of frequencies was performed using Fisher's exact test. Differences in percentage of MPO-positive blasts among patients with different mutational status of genes were compared using the non-parametric Kruskal-Wallis test and followed by Dunn's multiple comparison post-test. Overall survival (OS) was calculated using the Kaplan-Meier method [22], and the group differences were compared using the log-rank test. Thirteen patients who underwent allogeneic or autologous hematopoietic stem cell transplantation were not censored at the time of transplantation. For all analyses, statistical significance was considered at the level of two-tailed 0.05.

3 Results

3.1 Patients characteristics

As shown in Table 1, the series included 60 patients. Their median age was 59.5 (15-81 years), and there were 32 males (53.3%) and 28 females (46.7%). All patients had normal cytogenetics. Using the percentage of MPO positive leukemic blasts, as judged from bone marrow slides, the cases were divided into the High group (MPO positive blasts>50%) and Low group (MPO positive blasts≤50%). Thirty-two patients were classified into the Low group, and 28 patients were classified into the High group.

3.2 Mutational analysis

FLT3-ITD was found in 11 patients (18.3%), *NPM1* mutations were found in 19 patients (31.7%), and *CEBPA* mutations were found in 11 patients (18.3%). Frequency and an overlapping pattern of mutations are shown in Figure 1. Among the patients with *CEBPA* mutations, approximately 90% (10 of 11 patients) of the patients had two *CEBPA* mutations (*CEBPA*$^{\text{double-mut}}$), whereas 10% (1 of 11 patients) had a single mutation. As previously reported, the mutations in the *CEBPA*$^{\text{double-mut}}$ patients were clustered in the N- and C-terminal hotspots (Table 2, Figure 2). *FLT3*-ITD mutation was associated with a higher WBC at the time of diagnosis,

as reported previously. Neither *NPM1* nor *CEBPA* mutation status displayed a significant association with age, PS, WBC, FAB subtype, JALSG score, or CR achievement (Table 3).

3.3 Clinical outcome

OS was analyzed only in patients who received intensive chemotherapy (n=36). They received chemotherapy based on the treatment protocol described in the JALSG AML89, 92, 95, 97, and 201 studies. As reported previously [6], we observed an association between the percentage of MPO-positive blasts and the survival rate in the normal karyotype patients treated with intensive chemotherapy, although the significance in this cohort was rather low (p=0.10) (Figure 3). Figure 4 shows Kaplan-Meier curves according to genotype. 'Other genotypes' included the *FLT3*-ITD genotype, the *CEBPA* $^{\text{single-mut}}$ genotype, and the triple-negative genotype consisting of the wild-type *NPM1* and *CEBPA* genotypes without *FLT3*-ITD. In line with previous reports [14], the patients with the *CEBPA* $^{\text{double-mut}}$ genotype tended to show higher survival rate compared with patients displaying other genotypes (p=0.07). In this study, the mutant *NPM1* without *FLT3*-ITD genotype was not significantly associated with treatment outcome, possibly due to the small number of patients.

3.4 Difference of MPO-positivity rate by gene mutation status

Figure 5 shows the level of the percentage of MPO-positive blasts by gene mutational status of the *CEBPA*, *FLT3*-ITD, and *NPM1*. The MPO-positivity rate was very high, over 50% (median: 96, range: 71-100), in all *CEBPA* $^{\text{double-mut}}$ cases, but it was 20% in one case displaying the *CEBPA* $^{\text{single-mut}}$ genotype (data not shown). The MPO-positivity rate was widely distributed in patients who had mutant *NPM1* without *FLT3*-ITD genotype (median: 26, range: 0-100) and other genotypes (median: 31, range: 0-100). Kruskal-Wallis test showed that a significant difference of the MPO-positivity rate among three groups (P=0.005). When comparing the individual groups by Dunn's Multiple Comparisons post-hoc test for each group, there was a significant difference only for patients with *CEBPA* $^{\text{double-mut}}$ vs. patients with other genotypes.

4. Discussion

While cytogenetic group is considered to be the primary prognostic indicator in AML, the percentage of MPO-positive blast cells could be used to predict the prognosis of patients with normal karyotypes [6]. In this study, we found that *CEBPA* gene mutational status has impact on the frequency of MPO expression: the patients with the *CEBPA* mutation genotype displayed a significantly higher percentage of cells expressing MPO than those with other genotypes (P< 0.01). The association was even more significant when analyzed without the *CEBPA* $^{\text{single-mut}}$ carrying patient, suggesting that high blast MPO activity is related to double *CEBPA* mutations. Although the mutant *NPM1* without *FLT3*-ITD genotype has been reported to be

associated with a favorable prognosis in AML patients, there was no relationship between this type of mutation and the percentage of blasts showing MPO expression.

It is not clear how the *CEBPA*$^{\text{double-mut}}$ genotype enhances MPO activity in AML blasts. It has been shown that the MPO enhancer contains a site contributing to its functional activity [24], suggesting that the MPO gene is a major target of C/EBPα. Since it has been shown that both N-terminal frame–shift mutant and C-terminal mutant do not show transcriptional activity, we first speculated that mutations of the *CEBPA* gene might lead to decreased MPO activity, which turned out to be wrong. AML1 is another gene that has been reported to participate in up-regulation of MPO gene [25]. An AML1 site was identified in upstream enhancer of the human MPO gene, which appears to be necessary for maximal stimulation of MPO promoter activity. In patients with AML with t(8;21), the translocation results in an in-frame fusion between 5 exons of the AM1 gene and essentially all of the ETO gene producing a chimeric protein [26]. This protein, AML1-ETO, act as a negative dominant inhibitor of wild-type AML1 [27], which theoretically could lead to down-regulation of AML1 target genes, such as MPO gene. However, blasts with t(8;21) have been shown to display higher levels of MPO expression both in clinical samples and in vitro experiments [28,29], suggesting that the transcriptional alterations caused by these mutations are complex. The upregulation of blast MPO activity seen in CEBP/α$^{\text{double-mut}}$ cases may be due to alterations in the gene expression profile, rather than a simple dominant negative effect of mutated CEBP/α. Further experiments including investigation of transactivation potential of CEBP/α mutants on MPO promoter is necessary to clarify this mechanism.

CEBPA mutations are associated with a relatively favorable outcome, and it was recently shown in a multivariable analysis including cytogenetic risk and the *FLT3*-ITD and *NPM1* mutations that the *CEBPA*$^{\text{double-mut}}$ genotype is associated with favorable overall and event-free survival [15-16]. In a cohort of 60 cases of adult de novo AML, we identified 1 *CEBPA*$^{\text{single-mut}}$ case and 10 *CEBPA*$^{\text{double-mut}}$ cases, and in line with previous reports, our study tended to show better overall survival in *CEBPA*$^{\text{double-mut}}$ cases compared to cases with wild-type *CEBPA* in patients treated with intensive chemotherapy. We failed to find a prognostic effect in relation to the *CEBPA*$^{\text{double-mut}}$ in patients treated with low dose chemotherapy (data not shown), suggesting that the standard chemotherapy dose is necessary to improve the outcome of *CEBPA*$^{\text{double-mut}}$ cases.

It is unclear why *CEBPA*$^{\text{double-mut}}$ AML patients have a better outcome than those with *CEBPA* wild-type AML. One explanation is that high MPO expression leads to increased sensitivity to chemotherapeutic agents, such as to Ara-C [8]. To test this hypothesis, we also examined the association between blast MPO positivity and overall survival in *CEBPA* wild-type cases. Unexpectedly, when the patients were treated with intensive chemotherapy, the percentage of MPO-positive blasts was not significantly associated with overall survival in this group (data not shown), suggesting that the level of MPO expression itself is not responsible for the improvement in overall survival. However, as this analysis only involved 28 cases, we need to increase the number of cases in order to draw a definitive conclusion.

In summary, the data presented here suggested that the *CEBPA*$^{\text{double-mut}}$ genotype was associated with high MPO blast activity in patients with a normal karyotype. Although the results were obtained from a single institution, the presence of *CEBPA*$^{\text{double-mut}}$ genotype in high MPO group could explain at least in part why

high MPO blast activity is associated with better overall survival. Further studies in a larger cohort of patients are necessary to assess blast MPO activity as a prognostic factor, especially in *CEBPA* wild-type patients with a normal karyotype.

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Authors' Contributions

(1) the conception and design of the study, or acquisition of data, or analysis and interpretation of data; S T-S, K A, H I, Y I, J T, T F, S Y, T H, Y M

(2) drafting the article or revising it critically for important intellectual content; H T, D I,

(3) final approval of the version to be submitted; K K, H M, M T, Y M

Conflict of Interest

All authors have no conflict of interest to report.

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Figure legends

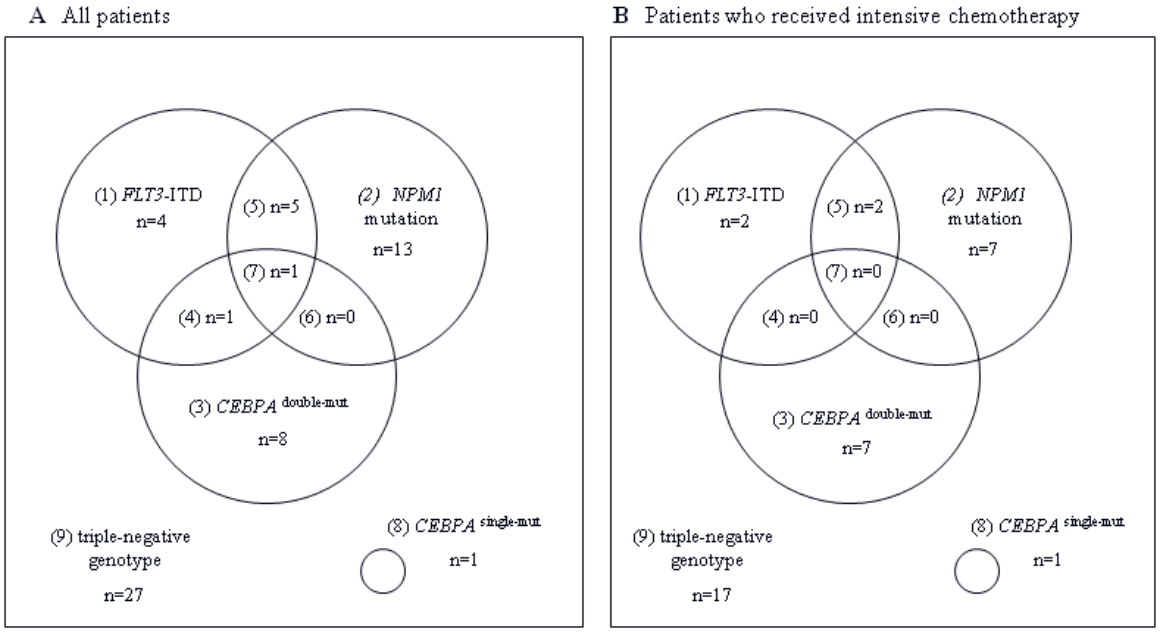

Fig. 1. Kaplan-Meier estimates of the probability of overall survival in 36 patients who received intensive chemotherapy, according to the percentage of myeloperoxidase-positive blasts. MPO-H (MPO positive blasts: >50%) tended to have a positive effect on overall survival compared with MPO-L (MPO positive blasts: ≤50%), although the difference was not statistically significant. The statistical significance of differences was evaluated with the log-rank test.

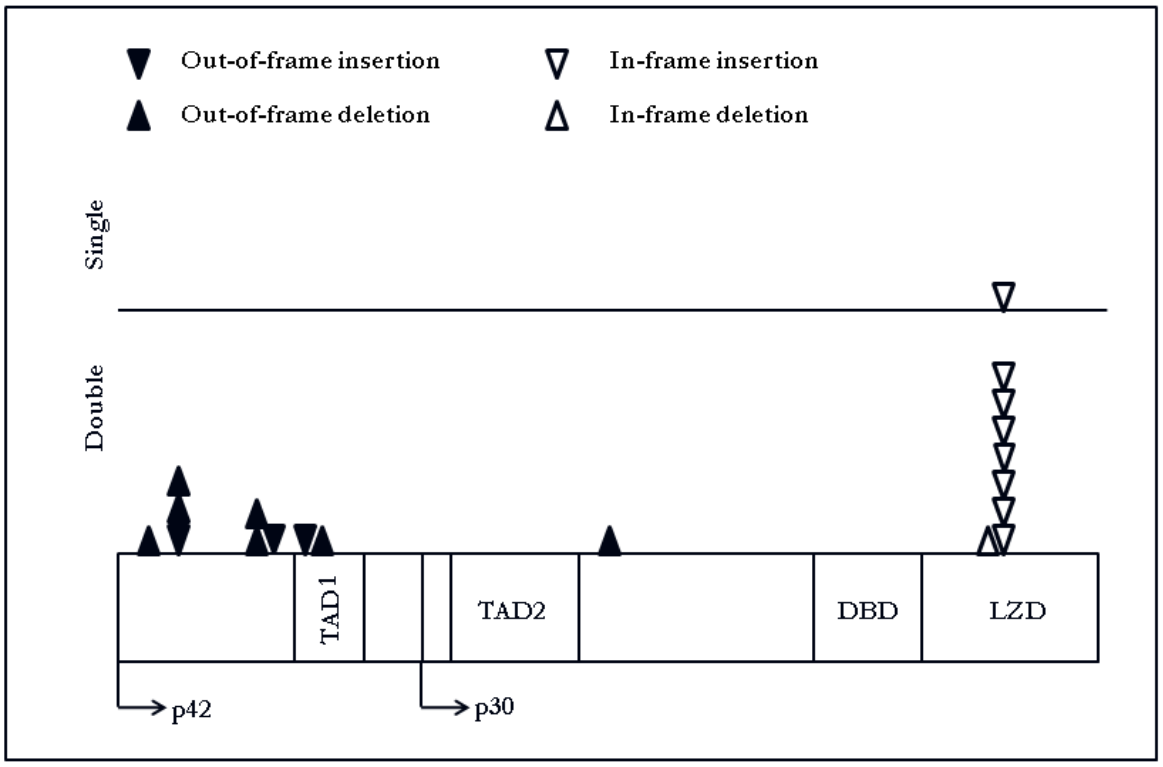

Fig. 2. Frequency and overlapping patterns of AML patients with a normal karyotype. Data are shown for all patients (panel A) and for patients who received intensive chemotherapy (panel B).

panel A: (1) *FLT3*-ITD+wt *NPM1*+wt *CEBPA* (n=4, 6.7%), (2) wt *FLT3*+*NPM1* mutation +wt *CEBPA* (n=13, 21.7%), (3) wt *FLT3*+wt *NPM1*+*CEBPA* $^{\text{double-mut}}$ (n=8, 13.3%), (4) *FLT3*-ITD+wt *NPM1*+*CEBPA* $^{\text{double-mut}}$ (n=1, 1.7%), (5) *FLT3*-ITD+*NPM1* mutation+wt *CEBPA* (n=5, 8.3%), (6) wt *FLT3*+*NPM1* mutation+*CEBPA* $^{\text{double-mut}}$ (n=0, 0%), (7) *FLT3*-ITD+*NPM1* mutation+*CEBPA* $^{\text{double-mut}}$ (n=1, 1.7%), (8) wt *FLT3*+wt *NPM1*+*CEBPA* $^{\text{single-mut}}$ (n=1, 1.7%), (9) triple-negative genotype (n=27, 45%)

panel B: (1) *FLT3*-ITD+wt *NPM1*+wt *CEBPA* (n=2, 5.6%), (2) wt *FLT3*+*NPM1* mutation+wt *CEBPA* (n=7, 19.4%), (3) wt *FLT3*+wt *NPM1*+*CEBPA* $^{\text{double-mut}}$ (n=7, 19.4%), (4) *FLT3*-ITD+wt *NPM1*+*CEBPA* $^{\text{double-mut}}$ (n=0, 0%), (5) *FLT3*-ITD+*NPM1* mutation+wt *CEBPA* (n=2, 5.6%), (6) wt *FLT3*+*NPM1* mutation+*CEBPA* $^{\text{double-mut}}$ (n=0, 0%), (7) *FLT3*-ITD+*NPM1* mutation+*CEBPA* $^{\text{double-mut}}$ (n=0, 0%), (8) wt *FLT3*+wt *NPM1*+*CEBPA* $^{\text{single-mut}}$ (n=0, 0%), (9) triple-negative genotype (n=17, 47.2%).

wt: wild-type

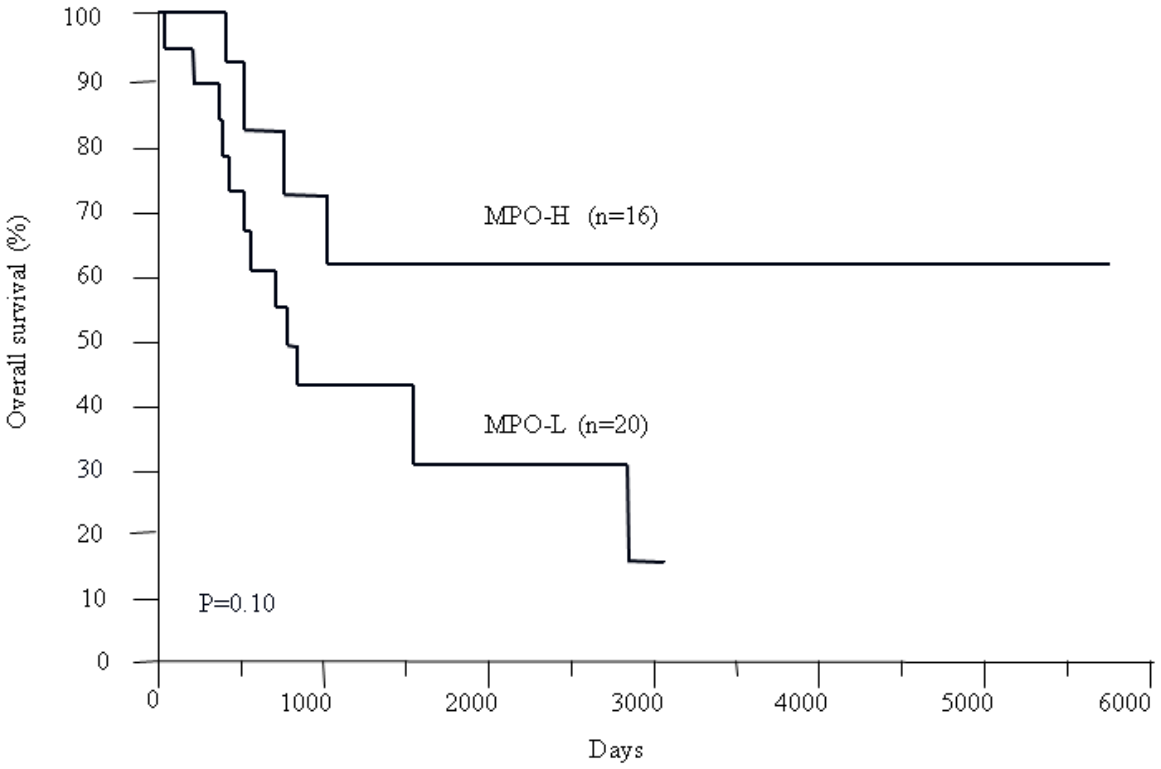

Fig. 3. Location of mutations detected in the *CEBPA* $^{\text{single-mut}}$ and *CEBPA* $^{\text{double-mut}}$ patients. Transactivation domain (TAD) 1, amino acids (AA) 70 to 97; p30 ATG, AA120; TAD2, AA 126 to 200; DNA-binding domain (DBD), AA 278 to 306; leucine zipper domain (LZD), AA 307 to 358.

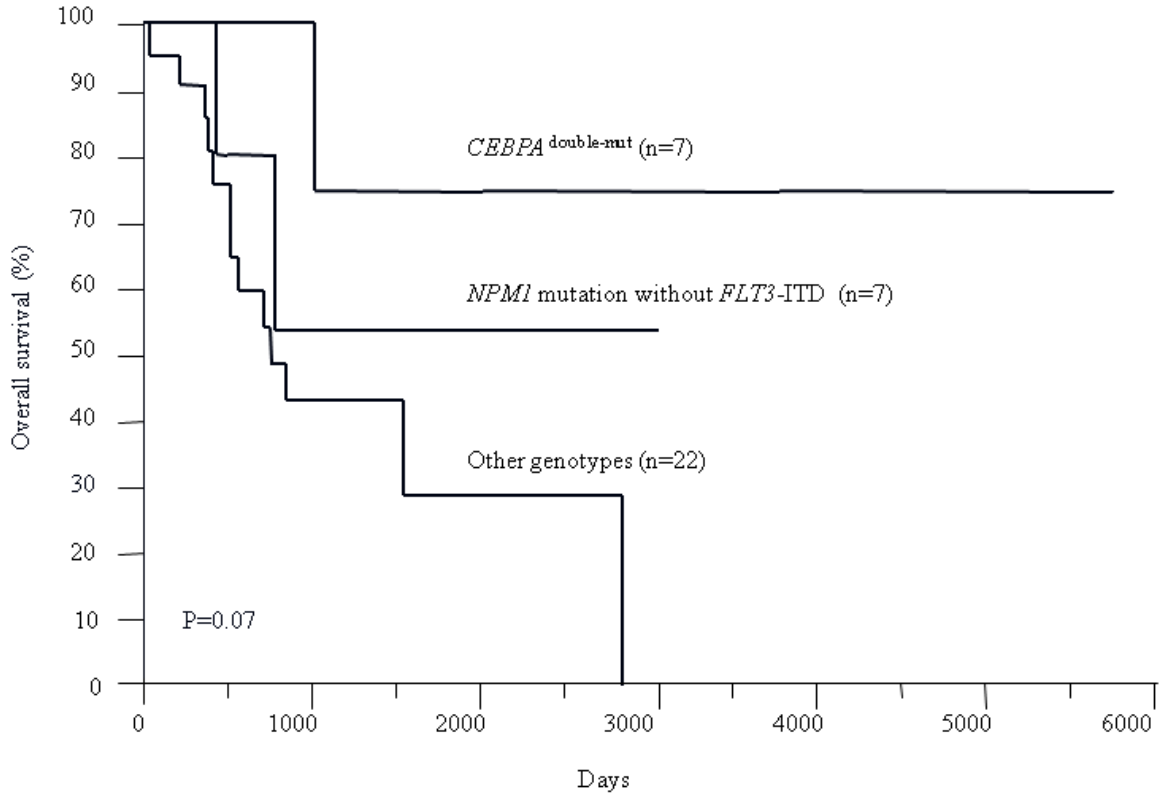

Fig. 4. Overall survival according to genotype in patients administered intensive chemotherapy. 'Other genotypes' was defined as the *FLT3*-ITD genotype, the *CEBPA* $^{\text{single-mut}}$ genotype, and the triple-negative genotype consisting of the wild-type *NPM1* and *CEBPA* genotypes without *FLT3*-ITD. The patients with the *CEBPA* $^{\text{double-mut}}$ genotype tended to show higher overall survival compared with the patients with 'other genotypes' (P=0.07).

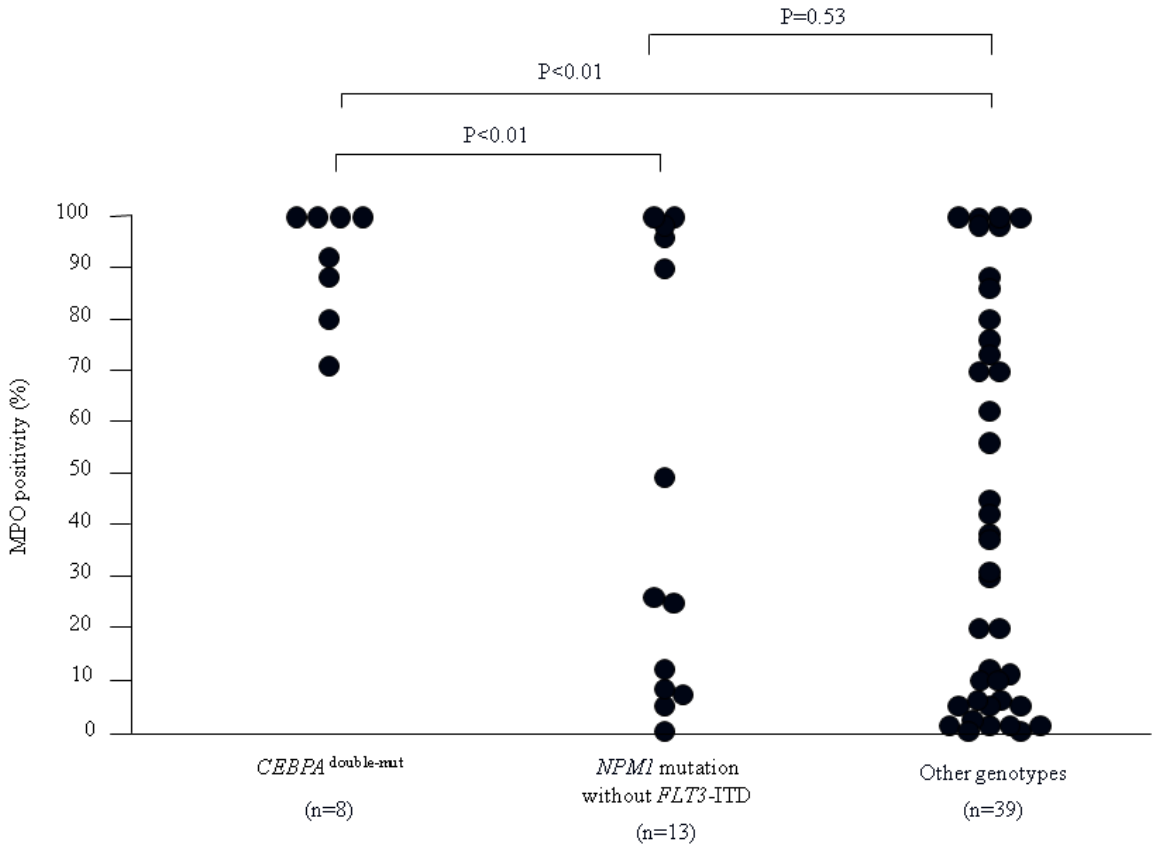

Fig. 5. MPO positivity rate in blast according to genetic abnormalities in de novo AML patients with a normal karyotype. 'Other genotypes' was defined as the *FLT3*-ITD genotype, the *CEBPA* $^{\text{single-mut}}$ genotype, and the triple-negative genotype consisting of the wild-type *NPM1* and *CEBPA* genotypes without *FLT3*-ITD. The median MPO positivity rate (horizon line) was significantly different between the *CEBPA* $^{\text{double-mut}}$ genotype and 'other genotypes' (Kruskal Wallis test followed by Dunn's multiple comparisons test: $P < 0.05$).

Table 1
Characteristics of de novo AML patients with a normal karyotype

	all patients (n=60)	patients who received intensive chemotherapy (n=36)
Median age (range) (year)	59.5 (15-81)	49 (15-67)
Male/female	32/28	18/18
FAB type		
M0	5	3
M1	10	5
M2	21	14
M4	18	11
M5	3	1
M6	3	2
M7	0	0
WBC ($\times 10^9$/l), median (range)	14.9 (0.7-556)	13.0 (0.7-246)
Performance status		
0-2	55	34
3-4	5	2
LDH (IU/l), median (range)	296 (120-5,325)	291 (140-2,606)
MPO		
Low (≦50%)	32	20
High (>50%)	28	16

FAB: French-American-British, WBC: white blood cells, LDH: lactate dehydrogenase, MPO: myeloperoxidase

Table 2

Genetic findings of the patients with *CEBPA* mutations.

Patient	Category	Nucleotide changes	Amino acid changes	Comments
4	double	218_219insC	P23fsX107	Produces N-terminal stop codon
		1129_1130insATGTGGAGACGCAGCAGAAGGTGCTGGAGCTGACCAGTGACAATGACCGCCTGCGCAAGC	K326_327insHVETQQKVLELTSDNDRLRKR	In-frame insertion in bZIP
6	double	200_218delinsCT	S16fsX101	Produces N-terminal stop codon
		1087_1089dup	K313dup	In-frame duplication in bZIP
7	double	368_369insA	A72fsX107	Produces N-terminal stop codon
		1080_1082del	T310_Q311del	In-frame deletion in bZIP
13	double	303_316del	P50fsX102	Produces N-terminal stop codon
		1062_1063insTTG	K304_Q305insV	In-frame insertion in bZIP
19	double	215_225del	P21fsX103	Produces N-terminal stop codon
		1101_1102insCAGCGCAACGTGGAGACGCAGCAGAAGGTGCTGGAGCTG	L317_T318insQRNVETQQKVLEL	In-frame insertion in bZIP
22	double	213del	P22fsX159	Produces N-terminal stop codon
		1064_1129dup	K304_Q305insQRNVETQQKVLELTSDNDRLRKR	In-frame insertion in bZIP
27	double	324_328dup	E59fsX161	Produces N-terminal stop codon
		1062_1063insTTG	K304_Q305insV	In-frame insertion in bZIP
39	double	213del	P22fsX159	Produces N-terminal stop codon
		1081-1086dup	Q311_Q312dup	In-frame duplication in bZIP
47	double	397del	F82fsX159	Produces N-terminal stop codon
		1101_1102insCAGCGCAACGTGGAGACGCAGCAGAAGGTGCTGGAGCTG	L317_T318insQRNVETQQKVLEL	In-frame insertion in bZIP
49	double	297_304del	A48fsX104	Produces N-terminal stop codon
		758del	A202fsX317	Frameshift between TAD2 and bZIP; produces stop codon in bZIP
35	single	1087_1089dup	K313dup	In-frame duplication in bZIP

bZIP: basic leucine zipper region, TAD2: second transactivation domain.

Nucleotide numbering was performed according to NCBI Entrez accession no. XM_009180.3, in which the major translational start codon starts at nucleotide position 151. The locations of functional domains are derived from Mueller and Pabst.1

Table 3

Relationships between *FLT3*-ITD, *NPM1*, and *CEBPA* mutations and clinical characteristics in de novo AML cases with a normal karyotype

	FLT3			*NPM1*			*CEBPA*		
	ITD	other type		mutation without *FLT3*-ITD	other type		double mutation without *FLT3*-ITD	other type	
	(n=11)	(n=49)	P value	(n=13)	(n=47)	P value	(n=8)	(n=52)	P value
Age			0.06			0.66			0.10
≦50	1	19		5	15		5	15	
＞50	10	30		8	32		3	37	
PS			0.92			0.30			0.52
0-2	10	45		11	45		7	48	
3-4	1	4		2	2		1	4	
WBC			0.01			0.97			1.00
≦20,000	2	30		7	25		4	28	
＞20,000	9	19		6	22		4	24	
FAB subtype			0.15			0.11			0.58
M1,M2,M4,M5	11	41		13	39		8	44	
M0,M6,M7	0	8		0	8		0	8	