Distinct gene alterations with a high percentage of myeloperoxidase-positive leukemic blasts in *de novo* acute myeloid leukemia

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Abstract

The myeloperoxidase (MPO)-positivity of blasts in bone marrow smears is an important marker for not only the diagnosis, but also the prognosis of acute myeloid leukemia (AML). To investigate the relationship between genetic alterations and MPO-positivity, we performed targeted sequencing for 51 genes and 10 chimeric gene transcripts in 164 newly diagnosed *de novo* AML patients; 107 and 57 patients were classified as AML with >50% MPO-positive blasts (MPO-high group) and ≤50% MPO-positive blasts, (MPO-low group), respectively. The univariate analysis revealed that *RUNX1-RUNX1T1* ($P < .001$), the *KIT* mutation ($P < .001$), and *CEBPA* double mutation ($P = .001$) were more likely to be found in the MPO-high group, while the *DNMT3A* mutation ($P = .001$), *FLT3* tyrosine kinase domain mutation ($P = .004$), and *TP53* mutation ($P = .020$) were more likely to be present in the MPO-low group. Mutations in genes related to DNA hypermethylation signatures (*IDH1*, *IDH2*, *TET2*, and *WT1* genes) were more frequent in the MPO-high group ($P = .001$) when patients with fusion genes of core-binding factors were excluded from the analysis. Our results suggest that MPO-positivity of blasts was related with the distinct gene mutation patterns among *de novo* AML patients.

Introduction

The expression of myeloperoxidase (MPO), a microbicidal protein, is a definitive marker for the diagnosis of acute myeloid leukemia (AML) in the French-American-British (FAB) and World Health Organization (WHO) classifications [1-3]. Although its expression in blasts (≥3%) represents a commitment to a myeloid lineage, the percentage of MPO-positive blasts varies widely among patients with AML. In the AML-92 and -201 clinical trials for AML patients conducted by the Japan Adult Leukemia Study Group (JALSG), patients with a high percentage of MPO-positive blasts had a significantly better outcome than those with a low percentage [4, 5]. Thus, the MPO-positivity of AML blasts is not only a lineage marker, but also a significant prognostic factor for AML patients. The MPO-positivity of blasts was also shown to have a significant impact on prognosis, even restricted to patients with a normal karyotype [4]. In vitro experiments also suggested that the expression of MPO in immature leukemia cells enhanced the sensitivity against cytarabine arabinoside [6]. Another clinical study, in which high levels of MPO mRNA in CD133-positive AML cells was significantly related with a better overall survival, further indicated that the MPO gene expression in immature leukemia cells could reflect the genetic and/or epigenetic profiles relating to the sensitivity against chemotherapy [7].

AML is highly heterogeneous for genetic and epigenetic alterations. Mutations in the *NPM1* and *CEBPA* genes are now markers of new disease entities for *de novo* AML in the WHO 2016 classification [3]. As indicated by recent studies, alterations in several genes are prognostic factors for patients treated with intensive chemotherapy (e.g. *FLT3*, *DNMT3A*, *TP53*, *MLL*, *NPM1*, and *CEBPA* genes) [8-10]. Genes coding epigenetic modifiers, spliceosome components, and cohesion complexes are also mutated in AML, and have been reported to affect its prognosis [11-13]. European LeukemiaNet (ELN) has recommended a risk stratification system based on the cytogenetic status and genetic alterations, such as *FLT3*, *NPM1*, and *CEBPA* genes [10]. In JALSG AML201 study, we have proposed that the overall survival of AML patients could be more clearly stratified by including the mutational status of *DNMT3A*, *MLL*-PTD, and *TP53* genes than the ELN system [12]. Accordingly, a comprehensive gene sequencing strategy is required to improve the quality of diagnoses, risk stratification, and treatment selection for AML.

We previously revealed that the *CEBPA* double mutation (*CEBPA* D-Mt) was identified only among AML patients with a high percentage of MPO-positive blasts [14], and we also demonstrated that the MPO-positivity of AML blasts correlated with distinct DNA methylation profiling [15]. These findings suggest the presence of a specific relationship between MPO-positivity and gene mutations in AML. Due to the crucial roles of MPO-

positivity and genetic alterations in the diagnosis and management of AML, it will be of interest to further investigate the relationship between gene mutation profiling and MPO-positivity. To address this issue, we performed a comprehensive analysis of genetic alterations in 51 genes and 10 fusion genes among 164 patients registered in the JALSG AML 201 study.

Patients and methods

Patients and samples

The present study included 164 adults with newly diagnosed *de novo* AML who were registered in the JALSG AML201 study (UMIN Clinical Trial Cord Registry C000000157). Patients with acute promyelocytic leukemia, a prior history of myelodysplastic syndromes, unexplained hematological abnormalities before the diagnosis of AML, a history of chemotherapy and/or radiation therapy, and exposure to toxic reagents were excluded.

A cytogenetic G-banding analysis was performed using standard methods and classified according to the Medical Research Council classification [16]. We also examined the presence of 10 chimeric gene transcripts (Major *BCR-ABL1*, Minor *BCR-ABL1*, *RUNX1-RUNX1T1*, *CBFB-MYH11*, *DEK-NUP214*, *NUP98-HOXA9*, *MLL-MLLT1*, *MLL-MLLT2*,

MLL-MLLT3, and *MLL-MLLT4*) by reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR) as previously reported [17].

We obtained informed consent from all patients in this study to use their samples (bone marrow cells) for a gene mutation analysis and banking. This study was approved by the Ethical Committee of each participating institute, and conducted in accordance with the Declaration of Helsinki.

Morphological central review

The Central Committee for Morphology of the JALSG reviewed slides of bone marrow and peripheral blood at diagnosis, and the morphological diagnosis of AML was reached based on the French-American-British (FAB) classification. The percentage of MPO-positive blasts was assessed by counting 100 blasts on bone marrow smears stained for MPO with the diaminobenzidine (DAB) method.

DNA sequencing of and a mutation analysis on 51 genes

High-molecular-weight DNA and total RNA were extracted from bone marrow samples using standard methods. A custom-made oligonucleotide probe library was designed to capture the exons of 51 genes that have been recurrently identified in myeloid neoplasms.

A detailed methodology and the panel of genes used in this study were previously reported [12]. In brief, captured and enriched exons were subjected to targeted sequencing on Illumina HiSeq (Illumina, San Diego, CA, USA) [12]. Sequence variation annotation was performed using the dbSNP database (Database of Single Nucleotide Polymorphisms) (https://www.ncbi.nlm.nih.gov/projects/SNP/snp_summary.cgi?build_id=131), followed by mutation characterization. Each predicted variant sequence was confirmed by Sanger sequencing. A mutational analysis for the internal tandem duplication of the *FLT3* gene (*FLT3*-ITD) and partial tandem duplication of the *MLL* gene (*MLL*-PTD) was performed as previously reported [18].

Grouping by MPO-positivity, gene categories, and gene alterations

AML patients were divided into two groups by the positivity of MPO enzymatic activity: AML patients with >50% MPO-positive blasts (MPO-high group) and those with ≤50% MPO-positive blasts (MPO-low group) [4-6, 14, 15]. Patients who had chromosomal abnormalities and/or the fusion genes of t(8;21)(q22;q22); *RUNX1-RUNX1T1* or inv(16)(p13q22); *CBFB-MYH11* were classified as core binding factor AML (CBF-AML).

Genetic alterations have been functionally classified into activated signaling gene

mutations (mutations in the *FLT3*, *KIT*, *K-RAS*, *N-RAS*, *PTPN11*, *JAK1*, and *JAK3* genes), which induce the constitutive activation of intracellular signals that contribute to growth and survival, and myeloid transcription factor gene mutations (mutations in the *CEBPA*, *GATA2*, *RUNX1*, and *ETV6* genes) that block differentiation and/or enhance self-renewal by altered factors. Genes that code proteins to control cell growth (*TP53*, *PHF6*, and *CBL* genes) were defined as tumor suppressor genes. Genes that code proteins related to DNA methylation (*DNMT3A*, *IDH1*, *IDH2*, *TET2*, and *WT1* genes) were defined as DNA methylation-related genes [19-22]. Genes that code proteins related to chromatin modifications (*KDM6A*, *MLL*, *MLL*-PTD, *DOT1L*, *ASXL1*, *ATRX*, *EZH2*, and *PBRM1* genes) were defined as chromatin modifiers [12, 23]. Other gene alterations were classified as NOTCH family genes (*NOTCH1* and *NOTCH2*), cohesion complex genes (*SMC1A*, *SMC3*, *STAG2*, and *RAD21*), BCOR family genes (*BCOR* and *BCORL1*), NCOR family genes (*NCOR1*, *NCOR2*, and *DIS3*), and spliceosome genes (*SF3B1*, *U2AF1*, *SRSF2*, and *ZRSR2*) [12].

In the JALSG AML 201 study [5], complete remission (CR) was defined as normal marrow cellularity with <5% blast cells with near-normal peripheral blood cell counts [24].

Statistical analysis

Differences in continuous variables were analyzed by the Wilcoxon rank-sum test for their distributions between two groups. The frequencies of gene mutations and the CR rate were analyzed by EZR (Saitama Medical Center, Jichi Medical University, Saitama, Japan) [25]. In all analyses, P-values were two-tailed, and a P-value of less than 0.05 was considered to indicate a significant difference.

Results

Patient characteristics by the percentage of MPO-positive blasts

Among 164 patients, 107 were classified into the MPO-high group and 57 into the MPO-low group (Table 1). A significant difference was observed in the distribution of AML subtypes according to the FAB classification between two groups (P<.001). AML cases in the MPO-high group were mainly classified as FAB-M2 (61.7%), while FAB-M4 was dominant in the MPO-low group (43.6%). The cytogenetic risk was significantly different between the MPO-high and -low groups (P <0.001). Fifty patients were diagnosed with CBF-AML; 48 had favorable karyotypes, such as t(8;21) or inv(16)/t(16;16), while 2 with normal karyotypes were classified as CBF-AML by the presence of a specific fusion gene (*CBFB-MYH11*). Among 104 cases with cytogenetic intermediate risk, both MPO-high

and -low patients were found (59 in MPO-high and 45 in MPO-low groups). Complex karyotypes with or without monosomy 7 or the partial deletion of the long arm of chromosome 7 were detected only in the MPO-low group. CR rates were significantly different between the two groups based on the MPO-positivity of blasts (Table 1, *P*=.013). In the analysis of patients with non-CBF-AML, a slight difference was observed in the CR rate between the two groups (*P* = .083): the CR rates of the MPO-high and -low groups were 83.1% and 67.3%, respectively.

Frequencies of gene alterations

Using target sequencing, mutations were detected in 44 out of the 51 genes tested, and RT-qPCR identified 6 fusion transcripts among 164 patients (Figure 1 and supplemental Table 1). No gene mutation or fusion transcript was detected in 3 patients in this study, 2 of whom had normal karyotypes. Each position and type of mutation have been reported previously [12]. We were unable to completely confirm whether all identified mutations were somatic because no germline material was available in this study. Accordingly, it was possible that a part of mutations could be rare SNPs. The frequencies of gene mutations were shown in supplemental Table 1, which was reported previously [12]. The mutations in the *CEBPA* gene were divided into *CEBPA* D-Mt (n=16, 9.8%) and the

CEBPA single mutation (*CEBPA* S-Mt) (n=10, 6.1%). The mutations in DNA methylation-related genes other than *DNMT3A* were identified in 28 patients (17.1%), including mutations in the *IDH1* in 9 (5.5%), *IDH2* in 8 (4.9%), *TET2* in 12 (7.3%), and *WT1* in 12 (7.3%).

Comparison of gene alterations by the MPO-positivity of blasts

No significant difference was observed in the number of mutated genes per patient between the MPO-high and MPO-low groups (P = .252) (supplemental Figure 1). The mean number of mutated genes was 2.61 ± 0.12 and 2.37 ± 0.19 in the MPO-high and -low groups, respectively. However, the profile of mutated genes and fusion transcripts differed by the MPO-positivity of blasts (Figure 2). A correlation was observed between CBF-fusion genes (i.e. *RUNX1-RUNX1T1* or *CBFB-MYH11* transcripts) and high MPO-positivity (P < .001), which was consistent with the relationship between favorable karyotypes and the MPO-high group. A mutation in the *KIT* gene also correlated with high MPO-positivity (P < .001). The presence of *KIT* mutations were mostly restricted to patients with CBF-AML, as previously shown (23 out of 26 cases). Mutations in the *CEBPA* gene were also significantly more frequent in the MPO-high group (25 out of 26 cases, P < .001). When single and double mutations were separately tested, *CEBPA* D-Mt,

but not *CEBPA* S-Mt, correlated with high MPO-positivity (P = .001). The presence of CBF-fusion genes and the *CEBPA* mutation was mutually exclusive among MPO-high patients. There were several gene mutations that correlated with low MPO-positivity; mutations in the *DNMT3A* (P = .001) and *TP53* (P = .020) genes, and *FLT3*-TKD (P = .004). Among AML patients in the MPO-low group, the *DNMT3A* mutation was mutually exclusive with the *TP53* mutation (see Figure 1).

Distinct gene mutations in patients with non-CBF-AML

As demonstrated above, in MPO-high group, CBF-fusion genes were the most frequent alteration. In terms of the overlap pattern with mutated genes based on the functional classification, mutations in activated signaling genes were frequently detected among cases with CBF-fusion genes (Figure 3), which was in good accordance with the "2-hit" genetic model of class I and class II [8, 18, 26]. On the contrary, among cases with non-CBF-AML, such genetic model was less consistent among non-CBF-AML cases, and the co-occurrence with DNA methylation-related gene mutation and other gene mutation tended to be more frequently observed. These results indicated the bias of genetic profiles of CBF-AML. To find the characteristic genetic alteration other than CBF-fusion genes, we next compared mutated genes and fusion transcripts with the positivity of MPO,

excluding CBF-AML patients. Among patients with non-CBF-AML, the number of mutated genes showed no significant difference regardless of the MPO-positivity of blasts (P=.490): 2.59 ± 0.17 and 2.42 ± 0.19 in the MPO-high and -low groups, respectively (supplemental Figure 2). The frequencies of mutated genes among these patients were as follows: mutations in the *FLT3* (n=36, 31.6%), *NPM1* (n=28, 24.6%), *CEBPA* (n=26, 22.8%), and *DNMT3A* (n=24, 21.1%) genes, which were identified in more than 10% of patients with non-CBF-AML. We then compared the frequency of gene alterations by the MPO-positivity of blasts (Figure 4). *CEBPA* D-Mt and *CEBPA* S-Mt both correlated with high MPO-positivity (P < .001 and P = .017, respectively), and the specificities of *CEBPA* D-Mt and *CEBPA* S-Mt were 100.0% and 98.1%, respectively, in these patients. A mutation in the *IDH2* gene was associated with high MPO-positivity (P = .062). In contrast, the frequency of *FLT3*-TKD was significantly higher in AML patients in the MPO-low group (P = .026), the specificity of which was 96.6%. An association was observed between the *DNMT3A* mutation and low MPO-positivity (P = .065), even among patients with non-CBF-AML. The specificity of the *DNMT3A* mutation was 86.4% in these patients.

We were interested in gene mutations related to DNA methylation because of our previous study on the relationship between the MPO-positivity of blasts and DNA

methylation profiles [15]. *IDH1/2*- or *TET2*-mutated hematopoietic cells display an overlapping DNA hypermethylation signature that correlates with a decrease in 5-hydroxymethylcytosine [19]. Moreover, the loss-of-function mutations in *WT1* gene has been reported to have the same consequence as loss-of-function mutations in *TET2* gene in terms of epigenetic regulation. Based on the previous and present studies showing the mutually exclusive mutations between *IDH1*, *IDH2*, *TET2*, and *WT1* in AML [29-30], we evaluated the relationship between the MPO-positivity of blasts and mutations in the *IDH1/2*, *TET2*, and *WT1* (*IDH1/2&TET2&WT1*) genes. Although no single mutation showed a significant relationship, mutations in the *IDH1/2&TET2&WT1* group correlated with high MPO-positivity (P = .001), the specificity of which was 83.6%. In the MPO-high group, 40 out of 59 patients had either the *CEBPA* or *IDH1/2&TET2&WT1* mutation; one patient had the *CEBPA* D-Mt and *TET2* mutations. *CEBPA* D-Mt and/or *IDH1/2&TET2&WT1* were mutated with 67.8% sensitivity and 83.6% specificity in these patients. The analysis for 4 chimeric gene transcripts (i.e. *DEK-NUP214*, *MLL-MLLT4*, *MLL-MLLT3*, and major *BCR-ABL1*) did not show any specific pattern based on the MPO-positivity of blasts.

Discussion

We herein focused on the relationship between the MPO-positivity of blasts by peroxidase staining and gene alterations using targeted sequencing. To the best of our knowledge, this is the first study to evaluate MPO-positivity as an indicator of the distinct pattern of gene alterations. The *RUNX1-RUNX1T1* fusion gene, *CEBPA* D-Mt, and *IDH1/2&TET2&WT1* mutations were more likely to be identified in the MPO-high group, whereas mutations in the *DNMT3A* and *TP53* genes were more likely to be identified in the MPO-low group. These results indicate that the MPO-positivity of blasts correlates with characteristic pattern of some gene alterations in patients with *de novo* AML.

The *RUNX1-RUNX1T1* fusion gene was the most frequently observed in the MPO-high group, which showed high specificity [2, 27]. AML with the *CBFB-MYH11* fusion gene is generally known to display high MPO-positivity [2], although our results did not show this due to the small number of patients with the *CBFB-MYH11* fusion gene. In addition, CBF-fusion genes were likely to co-occur with activated signaling gene mutations, such as *KIT* gene mutation, as reported previously [28-30], but this trend was less likely in non-CBF-AML patients. Provided that carefully designed prospective study confirmed the prognostic impact of activated signaling gene mutations in CBF-AML patients, our findings would further emphasize a high diagnostic value in the detection of the co-occurrence of both CBF-fusion genes and the mutations in activated signaling genes in

MPO-high group.

CEBPA D-Mt was identified in the MPO-high group with high specificity, which was mutually exclusive with CBF-AML. Our previous analysis also indicated that *CEBPA* D-Mt was associated with high MPO-positivity in AML patients having normal karyotypes [14]. Therefore, the present study using a comprehensive analysis further confirmed the relationship between *CEBPA* D-Mt and MPO-positivity. Moreover, because the WHO 2016 classification suggested the new entity "AML with *CEBPA* D-Mt" showing a favorable prognosis [3], screening for mutations in the *CEBPA* gene was considered for patients with high MPO-positivity, but not classified as CBF-AML.

The most interesting result of the present study was that MPO-positivity correlated with the mutations in DNA methylation-related genes. The *DNMT3A* mutation was significantly more prevalent in the MPO-low group. In contrast, the prevalence of *IDH1/2&TET2&WT1* mutations was higher in the MPO-high group when patients with CBF-AML were excluded. *DNMT3A* and *IDH1/2&TET2&WT1* seem biochemically opposite from the point of functions as DNA methylation machinery, and MPO-positivity of blast was reported to correlate with the distinct DNA methylation profile [15]. Based on these results, we speculate that the MPO-positivity would reflect the distinct DNA methylation status of leukemia cells, which could be induced by the variety of mutational

situations in DNA methylation-related genes. The presence of DNA methylation-related gene mutations may affect the selection of therapeutic strategies for patients with *de novo* AML in future. Several potent IDH1 and IDH2 mutant-targeting inhibitors (e.g. AG-221, AG-120, and IDH305) are currently being tested in clinical trials [31-33]. Furthermore, the *DNMT3A* mutation was reported to confer anthracycline resistance on AML cells [34]. Because it is possible that DNA methylation-related gene mutation will have an increasing impact on the selection of therapeutic option for AML in coming years, the attempt to the integrate the data about these gene mutational status into the traditional diagnostic modality, including the evaluation of MPO-positivity, should be required.

It is important to note that the MPO-low group was more likely to have the *TP53* mutation, which was associated with a poor prognosis in *de novo* AML treated with chemotherapy and transplantation. Previous studies revealed that the *TP53* mutation co-occurred in approximately 70% of *de novo* AML patients with adverse-risk karyotypes, such as the complex karyotype [13, 35, 36], but it can be detected in AML without adverse karyotype. Considering these situations, the mutational status in *TP53* genes should be carefully evaluated in the MPO-low group regardless of karyotype.

There were several limitations in the present study. We were unable to evaluate the impact of some mutated genes that were identified at lower frequencies in the present

study. An analysis of how the position and type of each gene mutation affected MPO-positivity was insufficient due to the small number of each mutation. Large-scale studies are warranted in the future to clarify the relationship between MPO-positivity and gene alterations in more detail.

In summary, MPO-positivity of blasts was significantly related to the underlying genetic alterations, such as CBF-fusion genes, DNA methylation-related genes, and mutations in *CEBPA* and *TP53*, among the patients with *de novo* AML. It remains elucidated regarding how these alterations affect the expression level of MPO in leukemia blasts and prognosis of AML.

Conflict of interest

HK received research funding from Chugai Pharmaceutical Co. Ltd., Bristol-Myers Squibb, Kyowa Hakko Kirin Co. Ltd., Zenyaku Kogyo Co., Ltd., FUJIFILM Corporation, Nippon Boehringer Ingelheim Co., Ltd., Astellas Pharma Inc., and Celgene Corporation, consulting fees from Astellas Pharma Inc. and Daiichi Sankyo Co.Ltd., and honoraria from Bristol-Myers Squibb and Pfizer.

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Author contributions

S Ogawa, TN, H Kiyoi, R Kamijo, and YM designed the study, interpreted the data and wrote the manuscript; R Kamijo, HI performed statistical analysis, interpreted the data and wrote the manuscript; R Kihara and YN performed molecular analysis and interpreted the data; YS, KC, HT, SM and S Ogawa performed bioinformatics; R Kamijo, TH, NA, and S Ohtake collected samples and clinical data. TH, NA, and S Ohtake also contributed to the interpretation of the data and critically reviewed the draft; and all authors approved the final version submitted for the publication.

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Author contributions

S Ogawa, TN, H Kiyoi, R Kamijo, and YM designed the study, interpreted the data and wrote the manuscript; R Kamijo, HI performed statistical analysis, interpreted the data and wrote the manuscript; R Kihara and YN performed molecular analysis and interpreted the data; YS, KC, HT, SM and S Ogawa performed bioinformatics; R Kamijo, TH, NA, and S Ohtake collected samples and clinical data. TH, NA, and S Ohtake also contributed to the interpretation of the data and critically reviewed the draft; and all authors approved the final version submitted for the publication.

Conflict of interest

HK received research funding from Chugai Pharmaceutical Co. Ltd., Bristol-Myers Squibb, Kyowa Hakko Kirin Co. Ltd., Zenyaku Kogyo Co., Ltd., FUJIFILM Corporation, Nippon Boehringer Ingelheim Co., Ltd., Astellas Pharma Inc., and Celgene Corporation, consulting fees from Astellas Pharma Inc. and Daiichi Sankyo Co.Ltd., and honoraria from Bristol-Myers Squibb and Pfizer.

Table 1. Patient characteristics

No. of cases (%)	MPO-high group	MPO-low group	P-value
Total	107	57	
Median age at diagnosis (range), y	46 (16-64)	51 (16-64)	0.410
Median WBC at diagnosis (/μl) (range)	16,600 (55-367,000)	17,900 (500-226,300)	0.940
FAB classification			<0.001
M0	0 (0.0)	5 (9.1)	
M1	19 (17.8)	6 (10.9)	
M2	66 (61.7)	16 (29.1)	
M4	21 (19.6)	24 (43.6)	
M5	1 (0.9)	5 (9.1)	
M6	0 (0.0)	1 (1.8)	
Cytogenetic risk classification			<0.001
Favorable risk group			
inv(16)(p13q22) / *CBFB-MYH11*	10 (9.3)	2 (3.5)	
t(8;21)(q22;q22) / *RUNX1-RUNX1T1*	38 (35.5)	0 (0.0)	
Intermediate risk group			
Normal	37 (34.6)	22 (38.6)	
Other	22 (20.6)	23 (40.4)	
Adverse risk group			
Complex with -7/del(7q)	0 (0.0)	4 (7.0)	
Complex without -7/del(7q)	0 (0.0)	5 (8.8)	
-7/del(7q)	0 (0.0)	1 (1.8)	
Other	0 (0.0)	0 (0.0)	
Response to induction therapy			0.013
CR	92 (86.0)	39 (68.4)	
Not achieving CR	15 (14.0)	18 (31.6)	

Abbreviations: AML, acute myeloid leukemia; MPO, myeloperoxidase; WBC, white blood cell counts; FAB classification, French-American-British classification; -7/del(7q), monosomy 7/partial deletion of 7q; CR, complete remission.

MPO-high group; AML with >50% MPO-positive blasts, MPO-low group; AML with ≤50% MPO-positive blasts.

Figure 1.

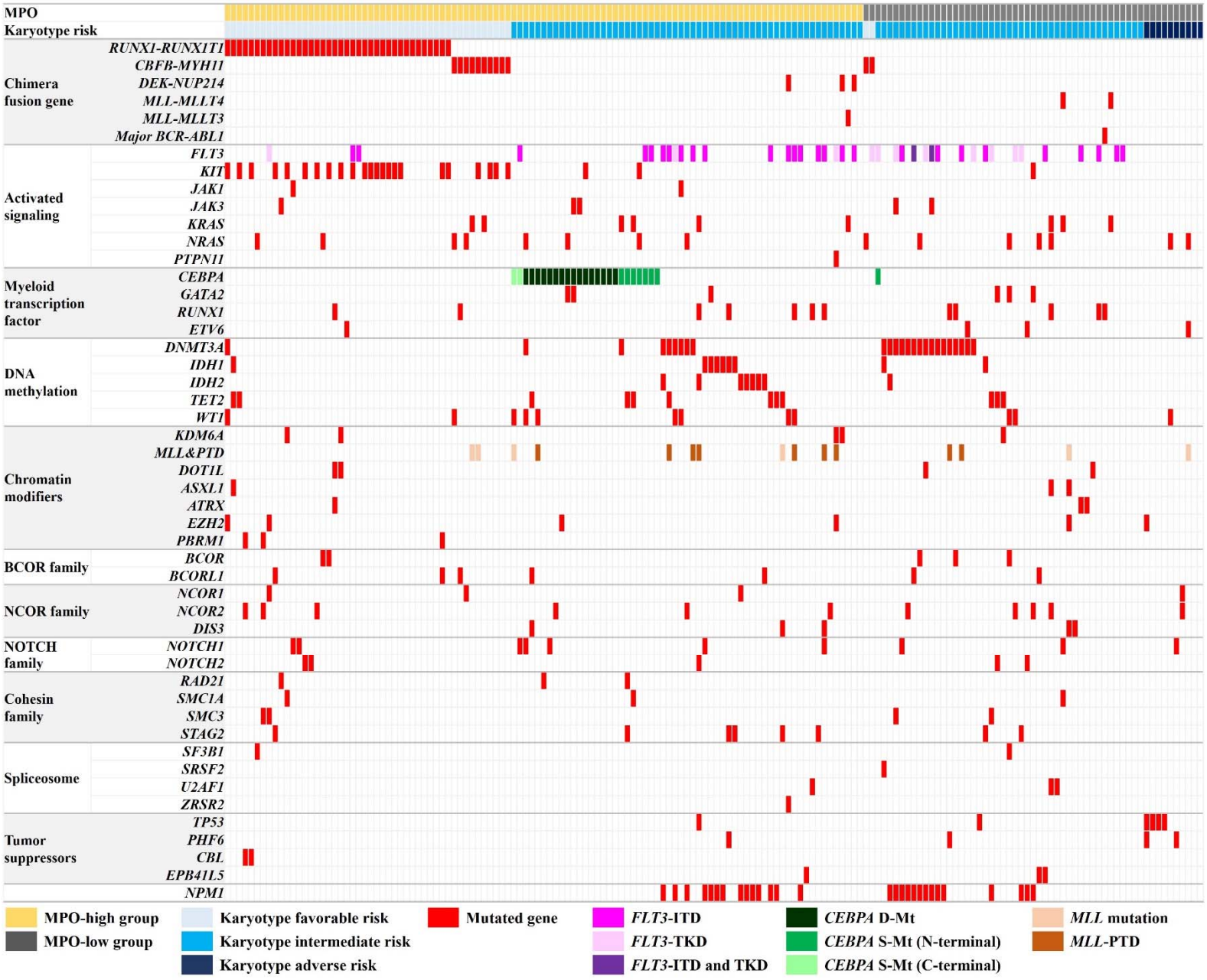
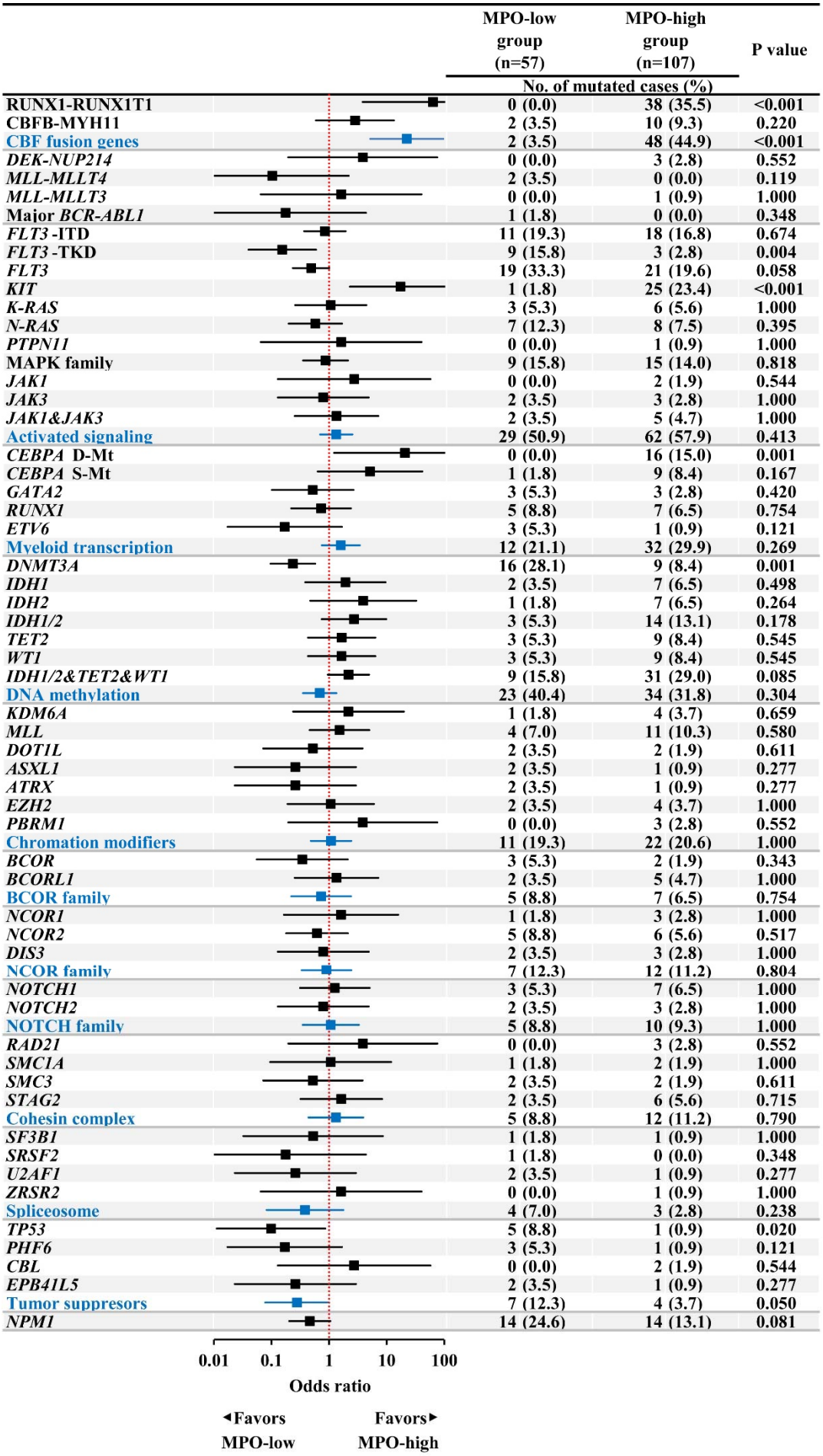

MPO
Karyotype risk
Chimera fusion gene
RUNX1-RUNX1T1
CBFB-MYH11
DEK-NUP214
MLL-MLLT4
MLL-MLLT3
Major BCR-ABL1
Activated signaling
FLT3
KIT
JAK1
JAK3
KRAS
NRAS
PTPN11
Myeloid transcription factor
CEBPA
GATA2
RUNX1
ETV6
DNA methylation
DNMT3A
IDH1
IDH2
TET2
WT1
Chromatin modifiers
KDM6A
MLL&PTD
DOT1L
ASXL1
ATRX
EZH2
PBRM1
BCOR family
BCOR
BCORL1
NCOR family
NCOR1
NCOR2
DIS3
NOTCH family
NOTCH1
NOTCH2
Cohesin family
RAD21
SMC1A
SMC3
STAG2
Spliceosome
SF3B1
SRSF2
U2AF1
ZRSR2
Tumor suppressors
TP53
PHF6
CBL
EPB41L5
NPM1
MPO-high group
MPO-low group
Karyotype favorable risk
Karyotype intermediate risk
Karyotype adverse risk
Mutated gene
FLT3-ITD
FLT3-TKD
FLT3-ITD and TKD
CEBPA D-Mt
CEBPA S-Mt (N-terminal)
CEBPA S-Mt (C-terminal)
MLL mutation
MLL-PTD

Figure 2.



	MPO-low group (n=57)	MPO-high group (n=107)	P value
	No. of mutated cases (%)		
RUNX1-RUNX1T1	0 (0.0)	38 (35.5)	<0.001
CBFB-MYH11	2 (3.5)	10 (9.3)	0.220
CBF fusion genes	2 (3.5)	48 (44.9)	<0.001
DEK-NUP214	0 (0.0)	3 (2.8)	0.552
MLL-MLLT4	2 (3.5)	0 (0.0)	0.119
MLL-MLLT3	0 (0.0)	1 (0.9)	1.000
Major *BCR-ABL1*	1 (1.8)	0 (0.0)	0.348
FLT3 -ITD	11 (19.3)	18 (16.8)	0.674
FLT3 -TKD	9 (15.8)	3 (2.8)	0.004
FLT3	19 (33.3)	21 (19.6)	0.058
KIT	1 (1.8)	25 (23.4)	<0.001
K-RAS	3 (5.3)	6 (5.6)	1.000
N-RAS	7 (12.3)	8 (7.5)	0.395
PTPN11	0 (0.0)	1 (0.9)	1.000
MAPK family	9 (15.8)	15 (14.0)	0.818
JAK1	0 (0.0)	2 (1.9)	0.544
JAK3	2 (3.5)	3 (2.8)	1.000
JAK1&JAK3	2 (3.5)	5 (4.7)	1.000
Activated signaling	29 (50.9)	62 (57.9)	0.413
CEBPA D-Mt	0 (0.0)	16 (15.0)	0.001
CEBPA S-Mt	1 (1.8)	9 (8.4)	0.167
GATA2	3 (5.3)	3 (2.8)	0.420
RUNX1	5 (8.8)	7 (6.5)	0.754
ETV6	3 (5.3)	1 (0.9)	0.121
Myeloid transcription	12 (21.1)	32 (29.9)	0.269
DNMT3A	16 (28.1)	9 (8.4)	0.001
IDH1	2 (3.5)	7 (6.5)	0.498
IDH2	1 (1.8)	7 (6.5)	0.264
IDH1/2	3 (5.3)	14 (13.1)	0.178
TET2	3 (5.3)	9 (8.4)	0.545
WT1	3 (5.3)	9 (8.4)	0.545
IDH1/2&TET2&WT1	9 (15.8)	31 (29.0)	0.085
DNA methylation	23 (40.4)	34 (31.8)	0.304
KDM6A	1 (1.8)	4 (3.7)	0.659
MLL	4 (7.0)	11 (10.3)	0.580
DOT1L	2 (3.5)	2 (1.9)	0.611
ASXL1	2 (3.5)	1 (0.9)	0.277
ATRX	2 (3.5)	1 (0.9)	0.277
EZH2	2 (3.5)	4 (3.7)	1.000
PBRM1	0 (0.0)	3 (2.8)	0.552
Chromation modifiers	11 (19.3)	22 (20.6)	1.000
BCOR	3 (5.3)	2 (1.9)	0.343
BCORL1	2 (3.5)	5 (4.7)	1.000
BCOR family	5 (8.8)	7 (6.5)	0.754
NCOR1	1 (1.8)	3 (2.8)	1.000
NCOR2	5 (8.8)	6 (5.6)	0.517
DIS3	2 (3.5)	3 (2.8)	1.000
NCOR family	7 (12.3)	12 (11.2)	0.804
NOTCH1	3 (5.3)	7 (6.5)	1.000
NOTCH2	2 (3.5)	3 (2.8)	1.000
NOTCH family	5 (8.8)	10 (9.3)	1.000
RAD21	0 (0.0)	3 (2.8)	0.552
SMC1A	1 (1.8)	2 (1.9)	1.000
SMC3	2 (3.5)	2 (1.9)	0.611
STAG2	2 (3.5)	6 (5.6)	0.715
Cohesin complex	5 (8.8)	12 (11.2)	0.790
SF3B1	1 (1.8)	1 (0.9)	1.000
SRSF2	1 (1.8)	0 (0.0)	0.348
U2AF1	2 (3.5)	1 (0.9)	0.277
ZRSR2	0 (0.0)	1 (0.9)	1.000
Spliceosome	4 (7.0)	3 (2.8)	0.238
TP53	5 (8.8)	1 (0.9)	0.020
PHF6	3 (5.3)	1 (0.9)	0.121
CBL	0 (0.0)	2 (1.9)	0.544
EPB41L5	2 (3.5)	1 (0.9)	0.277
Tumor suppresors	7 (12.3)	4 (3.7)	0.050
NPM1	14 (24.6)	14 (13.1)	0.081

Figure 3.

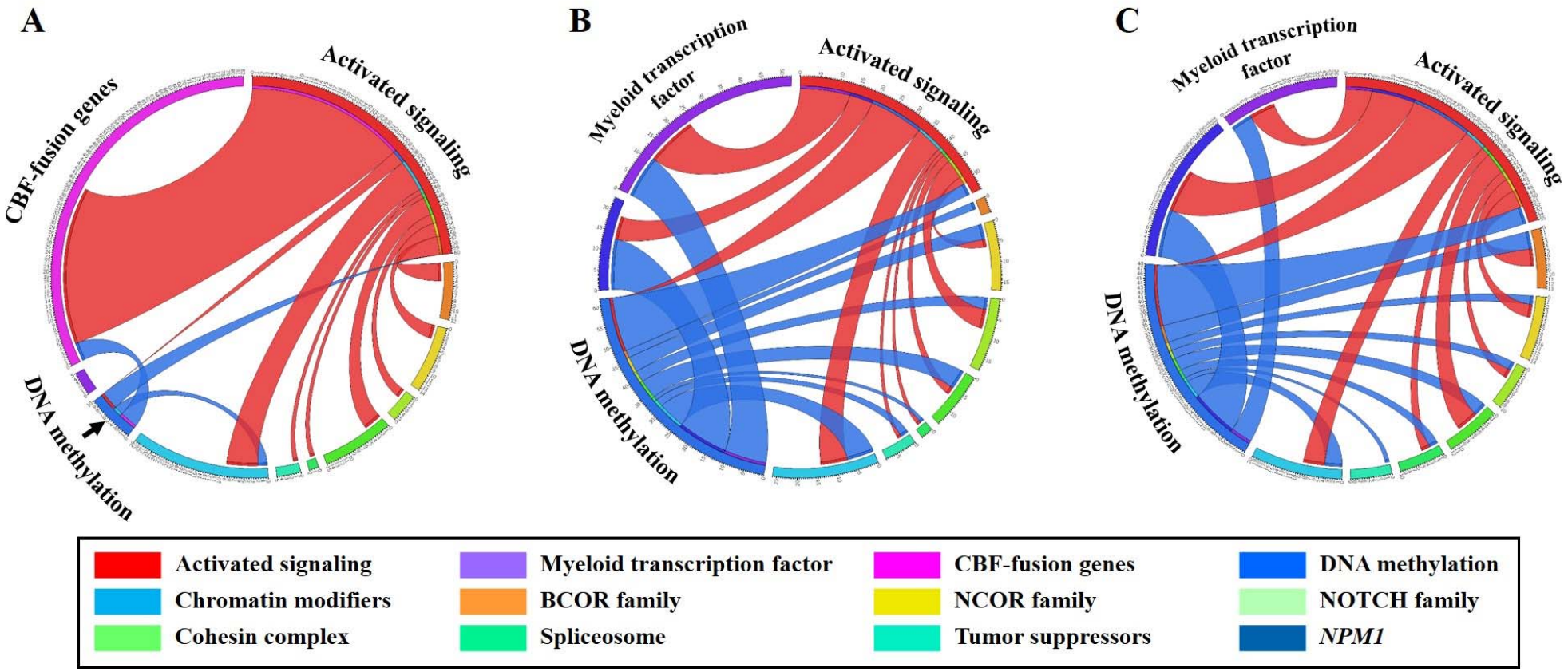
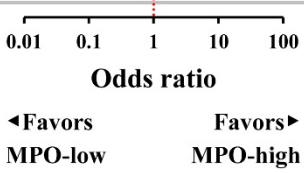

A
Activated signaling
CBF-fusion genes
DNA methylation
B
Myeloid transcription factor
Activated signaling
DNA methylation
C
Myeloid transcription factor
Activated signaling
DNA methylation
Activated signaling
Chromatin modifiers
Cohesin complex
Myeloid transcription factor
BCOR family
Spliceosome
CBF-fusion genes
NCOR family
Tumor suppressors
DNA methylation
NOTCH family
NPM1

Figure 4.

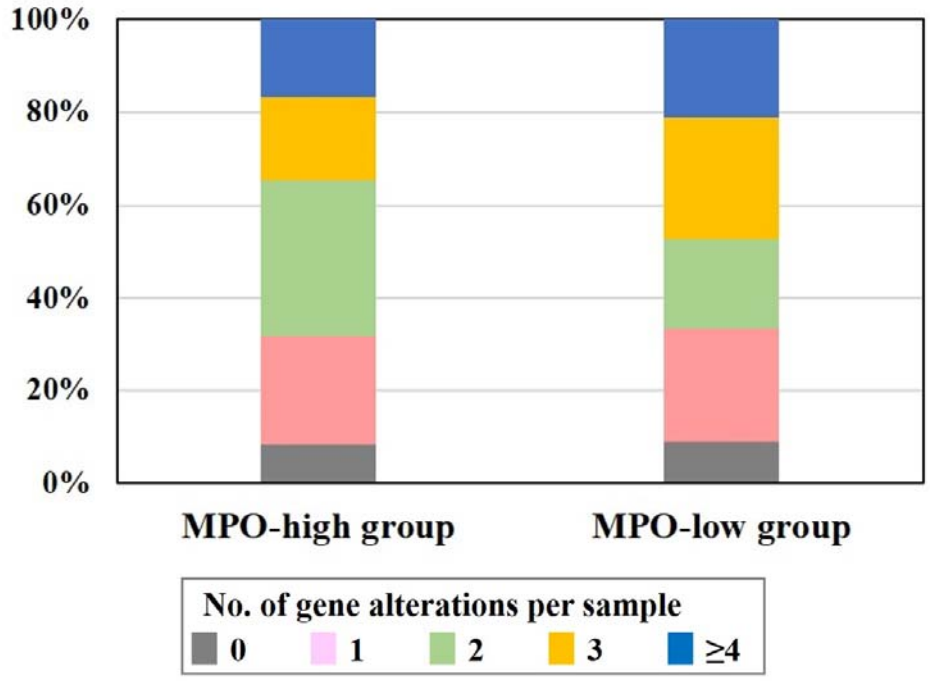


	MPO-low group (n=55)	MPO-high group (n=59)	P value
	No. of mutated cases (%)		
DEK-NUP214	0 (0.0)	3 (5.1)	0.244
MLL-MLLT4	2 (3.6)	0 (0.0)	0.231
MLL-MLLT3	0 (0.0)	1 (1.7)	1.000
Major *BCR-ABL1*	1 (1.8)	0 (0.0)	0.483
FLT3 -ITD	11 (20.0)	16 (27.1)	0.389
FLT3 -TKD	9 (16.4)	2 (3.4)	0.026
FLT3	18 (32.7)	18 (30.5)	0.842
KIT	1 (1.8)	2 (3.4)	1.000
K-RAS	3 (5.5)	4 (6.8)	1.000
N-RAS	6 (10.9)	4 (6.8)	0.518
PTPN11	1 (1.8)	0 (0.0)	0.483
MAPK family	9 (16.4)	8 (13.6)	0.794
JAK1	0 (0.0)	1 (1.7)	1.000
JAK3	2 (3.6)	2 (3.4)	1.000
JAK1&JAK3	2 (3.6)	3 (5.1)	1.000
Activated signaling	27 (49.1)	29 (49.2)	1.000
CEBPA D-Mt	0 (0.0)	16 (27.1)	<0.001
CEBPA S-Mt	1 (1.8)	9 (15.3)	0.017
GATA2	3 (5.5)	3 (5.1)	1.000
RUNX1	5 (9.1)	5 (8.5)	1.000
ETV6	3 (5.5)	0 (0.0)	0.109
Myeloid transcription	12 (21.8)	29 (49.2)	0.003
DNMT3A	16 (29.1)	8 (13.6)	0.065
IDH1	2 (3.6)	6 (10.2)	0.274
IDH2	1 (1.8)	7 (11.9)	0.062
IDH1/2	3 (5.5)	13 (22.0)	0.014
TET2	3 (5.5)	7 (11.9)	0.324
WT1	3 (5.5)	7 (11.9)	0.324
IDH1/2&TET2&WT1	9 (16.4)	27 (45.8)	0.001
DNA methylation	23 (41.8)	30 (50.8)	0.354
KDM6A	1 (1.8)	2 (3.4)	1.000
MLL	4 (7.3)	9 (15.3)	0.242
DOT1L	2 (3.6)	0 (0.0)	0.231
ASXL1	2 (3.6)	0 (0.0)	0.231
ATRX	2 (3.6)	0 (0.0)	0.231
EZH2	2 (3.6)	2 (3.4)	1.000
PBRM1	0 (0.0)	0 (0.0)	-
Chromation modifiers	11 (20.0)	11 (18.6)	1.000
BCOR	3 (5.5)	0 (0.0)	0.109
BCORL1	2 (3.6)	2 (3.4)	0.109
BCOR family	5 (9.1)	2 (3.4)	0.260
NCOR1	1 (1.8)	1 (1.7)	1.000
NCOR2	5 (9.1)	3 (5.1)	0.479
DIS3	2 (3.6)	3 (5.1)	1.000
NCOR family	7 (12.7)	7 (11.9)	0.923
NOTCH1	3 (5.5)	5 (8.5)	0.718
NOTCH2	3 (5.5)	1 (1.7)	0.351
NOTCH family	5 (9.1)	6 (10.2)	1.000
RAD21	0 (0.0)	2 (3.4)	0.496
SMC1A	1 (1.8)	1 (1.7)	1.000
SMC3	2 (3.6)	0 (0.0)	0.231
STAG2	2 (3.6)	5 (8.5)	0.440
Cohesin complex	5 (9.1)	7 (11.9)	0.764
SF3B1	1 (1.8)	0 (0.0)	0.483
SRSF2	1 (1.8)	0 (0.0)	0.483
U2AF1	2 (3.6)	1 (1.7)	0.609
ZRSR2	0 (0.0)	1 (1.7)	1.000
Spliceosome	4 (7.3)	2 (3.4)	0.427
TP53	5 (9.1)	1 (1.7)	0.105
PHF6	3 (5.5)	1 (1.7)	0.351
CBL	0 (0.0)	0 (0.0)	-
EPB41L5	2 (3.6)	1 (1.7)	0.609
Tumor suppresors	7 (12.7)	2 (3.4)	0.086
NPM1	14 (25.5)	14 (23.7)	1.000

Supplemental Table 1. Prevalence of gene mutations

Category	Gene mutations	Total (n=164)	
		No. of cases	(%)
Activated signaling	*FLT3*-ITD	29	(17.7)
	FLT3-TKD	13	(7.9)
	FLT3	39	(23.8)
	KIT	26	(15.9)
	K-RAS	9	(5.5)
	N-RAS	15	(9.1)
	PTPN11	1	(0.6)
	MAPK family	24	(14.6)
	JAK1	2	(1.2)
	JAK3	5	(3.0)
	JAK1&JAK3	7	(4.3)
Myeloid transcription factor	*CEBPA* D-Mt	16	(9.8)
	CEBPA S-Mt	10	(6.1)
	GATA2	6	(3.7)
	RUNX1	12	(7.3)
	ETV6	4	(2.4)
DNA methylation	*DNMT3A*	25	(15.2)
	IDH1	9	(5.5)
	IDH2	8	(4.9)
	IDH1&IDH2	17	(10.4)
	TET2	12	(7.3)
	WT1	12	(7.3)
	IDH1/2&TET2&WT1	40	(24.4)
Chromosomal modifiers	*KDM6A*	5	(3.0)
	MLL	6	(3.7)
	DOT1L	4	(2.4)
	ASXL1	3	(1.8)
	ATRX	3	(1.8)
	EZH2	6	(3.7)
BCOR family	*BCOR*	5	(3.0)
	BCORL1	7	(4.3)
NCOR family	*NCOR1*	4	(2.4)
	NCOR2	11	(6.7)
	DIS3	5	(3.0)
NOTCH family	*NOTCH1*	10	(6.1)
	NOTCH2	6	(3.7)
Cohesin complex	*RAD21*	3	(1.8)
	SMC1A	3	(1.8)
	SMC3	4	(2.4)
	STAG2	8	(4.9)
Spliceosome	*SF3B1*	2	(1.2)
	SRSF2	1	(0.6)
	U2AF1	3	(1.8)
	ZRSR2	1	(0.6)
Tumor suppressors	*TP53*	6	(3.7)
	PHF6	4	(2.4)
	CBL	2	(1.2)
	EPB41L5	3	(1.8)
Other	*NPM1*	28	(17.1)

Supplemental Figure 1. Number of gene alternations in each group



Supplemental Figure 2. Number of gene alternations in each group when CBF-AML was excluded

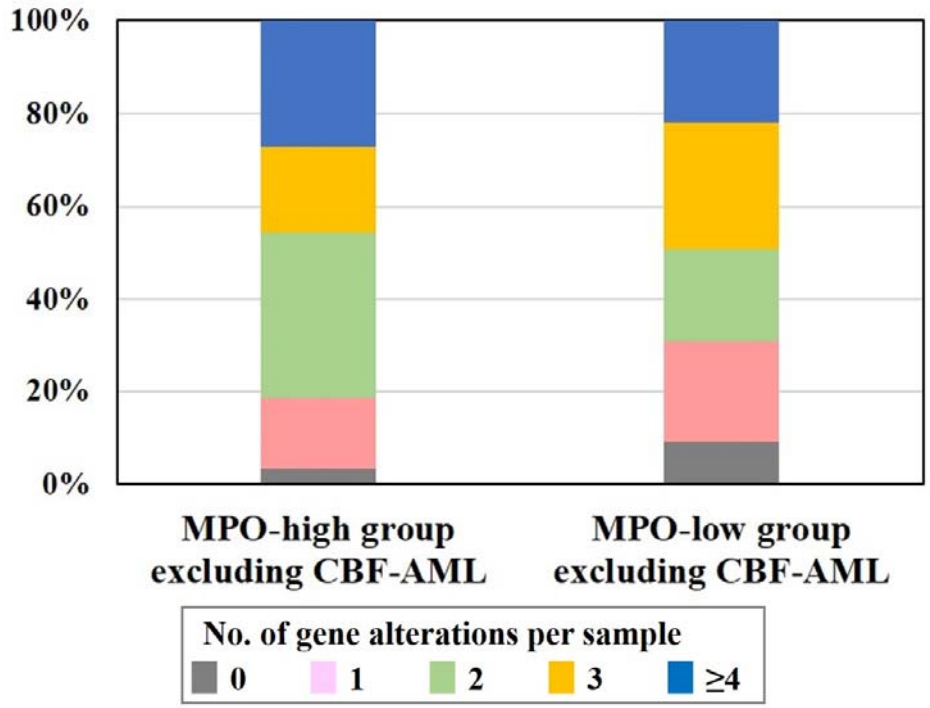


Figure Legends

Figure 1. Mutational status based on MPO positivity

Abbreviations: *FLT3*-ITD, internal tandem duplication of the *FLT3* gene; *FLT3*-TKD, *FLT3* tyrosine kinase domain mutation; *CEBPA* D-Mt, *CEBPA* double mutation; *CEBPA* S-Mt, *CEBPA* single mutation, *MLL*-PTD, partial tandem duplication of the *MLL* gene.

Figure 2. Relationship between individual mutated genes and the MPO positivity of blasts.

Forest plot; Blue lines indicate the functional classification of gene alterations. The number and frequency of cases with mutations in each gene by the MPO positivity of blasts are show on the right. *JAK* family members are tyrosine kinases that stimulate cell proliferation via the JAK-STAT pathway.

KRAS, *NRAS*, and *PTPN11* are members of the Ras-mitogen activated protein kinase (MAPK) signaling pathway family. The mutations in *IDH1/2*, *TET2*, and *WT1* genes was grouped into similar regulator in terms of DNA machinery.

Figure 3. Co-occurrence pattern of genes alterations according to the functional classification.

Co-occurrence of mutation is represented by lines connecting genes [37]. Circos plots of gene alterations are shown: (A) CBF-AML patients, (B) the MPO-high group excluding CBF-AML, and (C) the MPO-low group excluding CBF-AML. The width of connecting lines represents frequency of gene alterations. Activated signaling gene mutations (red ribbons) and DNA methylation-related gene alterations (blue ribbons) are combined in this analysis.

Figure 4. Relationship between individual mutated genes and the MPO positivity of blasts in patients with non-CBF-AML.

This analysis did not include patients with CBF-AML.

Supplemental Figure 1. Number of gene alternations in each group.

The number of mutated genes per sample by the MPO positivity of blasts in the MPO-high group and MPO-

low group.

Supplemental Figure 2. Number of gene alternations in each group when CBF-AML was excluded.

The number of mutated genes per sample by the MPO positivity of blasts in the MPO-high group and MPO-low group when patients with core binding factor acute myeloid leukemia (CBF-AML) were excluded.