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Research paper

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* ($P < 0.001$), the *KIT* mutation ($P < 0.001$), and *CEBPA* double mutation ($P = 0.001$) were more likely to be found in the MPO-high group, while the *DNMT3A* mutation ($P = 0.001$), *FLT3* tyrosine kinase domain mutation ($P = 0.004$), and *TP53* mutation ($P = 0.020$) 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 ($P = 0.001$) 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.

1. 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

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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.

2. Patients and methods

2.1. 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.

2.2. 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.

2.3. 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].

2.4. 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 $\leq 50\%$ 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].

2.5. 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.

3. Results

3.1. 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 ($P < 0.001$). AML cases in the MPO-