Gene mutation patterns and their prognostic impact in a cohort of 1185 patients with acute myeloid leukemia

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To evaluate the prognostic value of genetic mutations for acute myeloid leukemia (AML) patients, we examined the gene status for both fusion products such as *AML1* (*CBFα*)–*ETO*, *CBFβ-MYH11*, *PML-RARα*, and *MLL* rearrangement as a result of chromosomal translocations and mutations in genes including *FLT3*, *C-KIT*, *N-RAS*, *NPM1*, *CEBPA*, *WT1*, *ASXL1*, *DNMT3A*, *MLL*, *IDH1*, *IDH2*, and *TET2* in 1185 AML patients. Clinical analysis was mainly carried out among 605 cases without recognizable karyotype abnormalities except for 11q23. Of these 605 patients, 452 (74.7%) were found to have at least 1 mutation, and the relationship of gene mutations with clinical outcome was investigated. We revealed a correlation pattern among *NPM1*, *DNMT3A*, *FLT3*, *IDH1*, *IDH2*, *CEBPA*, and *TET2* mutations. Multivariate analysis identified *DNMT3A* and *MLL* mutations as independent factors predicting inferior overall survival (OS) and event-free survival (EFS), whereas biallelic *CEBPA* mutations or *NPM1* mutations without *DNMT3A* mutations conferred a better OS and EFS in both the whole group and among younger patients < 60 years of age. The use of molecular markers allowed us to subdivide the series of 605 patients into distinct prognostic groups with potential clinical relevance. (*Blood*. 2011;118(20):5593-5603)

Introduction

Acute myeloid leukemia (AML) is a group of heterogeneous diseases with considerable diversity in terms of clinical behavior and prognosis.[1-5] Clinical and genetic prognostic markers are now crucial in the evaluation of AML patients and in guiding rational management. Among them, cytogenetic abnormalities are considered to be the most important prognostic factors of AML.[5-11] For example, acute promyelocytic leukemia (APL, AML-M3) with t(15;17) translocation is associated with a favorable prognosis,[12-14] and core-binding factor (*CBF*) leukemias with t(8;21) translocation in AML-M2 variant (M2v) or inv(16) rearrangement in AML-M4 with eosinophilia (M4eo) have also been reported to have relatively good outcome.[5,7,15] Nevertheless, ~ 1/2 of AML patients lack typical prognostic karyotypic changes. To improve clinical outcome, it is important to identify specific and accurate predictors in this group of patients using molecular approaches.

It has been proposed that, according to their roles in pathogenesis, genetic abnormalities in leukemia can be roughly grouped into 2 classes: (1) mutations involving signal transduction pathways and giving rise to proliferative advantages to leukemia clones (class I) and (2) those affecting transcription factors or cofactors and causing impaired differentiation (class II).[16]Numerous gene mutations have been discovered in AML patients without cytogenetic markers, and these abnormalities have been considered to belong to either class I, as exemplified by internal tandem duplications (ITDs) or mutations of the tyrosine kinase domain (TKD) of both *C-KIT*[17,18] and FMS-like tyrosine kinase 3 (*FLT3*) genes[11,19,20] and point mutations of the neuroblastoma RAS viral oncogene homolog gene (*NRAS*)[17,21-24] or to class II, as exemplified by mutations of nucleophosmin gene (*NPM1*),[2,4,22,25] the CCAAT/enhancer binding protein α gene (*CEBPA*), the Wilm tumor (*WT1*) gene, and the additional sex combs like 1 (*ASXL1*) gene.[26-30] Recently, a new category of gene mutations associated with epigenetic regulation have drawn much attention, including the mutations of isocitrate dehydrogenase1 (*IDH1*), isocitrate dehydrogenase2 (*IDH2*), and ten-eleven-2 (*TET2*), which result in a hypermethylation phenotype with impairment of hematopoietic differentiation.[31-33] The mixed-lineage leukemia (*MLL*) gene, which can be affected either through chromosomal translocation or via an intragenic partial tandem duplications (PTDs) to form a fusion gene, actually encodes a histone methyltransferase.[22,34-36] The discovery of these genetic events has raised the possibility of a new class of leukemogenic genes.

The above-mentioned molecular aberrations exert profound effects on individual response to the therapy and treatment outcome of the disease.[14,16,37] It has been described that *CEBPA* mutations[27,28] and *NPM1* mutations without *FLT3*-ITD[16,20,23,25] are associated with a favorable prognosis, whereas gene mutations associated with a poor prognosis include *C-KIT* involvement among *CBF* AMLs,[17,18,23] *FLT3*-ITD without *NPM1* mutations,[16,20,23,25] and *MLL*-PTD mutations among AML with normal cytogenetics.[22,34,35] However, the role of leukemic *IDH1* and *IDH2* mutations and *WT1*, *TET2*, and *ASXL1* mutations in predicting the prognosis of AML are not clearly established.[31-33,38] In addition, these markers may also provide potential molecular targets for

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tailored therapies, as recently reported by several groups demonstrating potential clinical values of sorafenib in the patients with *FLT3* ITD and TKD, and azacitidine and decitabine in patients with *MLL* abnormalities.[39]

More recently, genomic sequencing research in AML and other malignancies has greatly facilitated the identification of new oncogenic mutations. We[40] and others[37] have discovered mutations in the DNA methyltransferase 3A (*DNMT3A*) gene in $> 20\%$ of acute monocytic leukemia patients using exome sequencing and subsequent Sanger sequencing. The enzyme encoded by the *DNMT3A* gene is responsible for de novo DNA cytosine methylation.[37,41-43] Both studies suggested that *DNMT3A* mutations are associated with hyperleukocytosis at disease presentation, elderly age, and poor prognosis. With the accumulation of more new data, decision making for risk-stratified therapy will be possible and should be integrated into the individualized treatment of AML.

We performed this study to systemically investigate the frequencies and the prognostic relevance of previously known genetic events and newly established molecular markers in a large series of adult AML patients. In particular, our intent was to stratify the "ambiguous" AML patients who lack cytogenetic prognostic markers into appropriate prognostic groups using molecular markers.

Methods

Patients

BM and peripheral blood samples were collected from 1185 patients with de novo AML from 1998-2010 from the centers of Shanghai Institute of Hematology (SIH) and Zhejiang Institute of Hematology (ZIH). French-American-British (FAB) criteria were used to define the AML subtypes (M0 through M7, with a few cases not classifiable according to the FAB nomenclature). Patients with leukemia either transformed from myelodysplastic syndrome or secondary to other malignancies were excluded from this study.

All the samples were assessed for overview of pattern and distribution of gene mutations, and they were further divided into 3 groups. Group I, containing 605 patients without prognostic cytogenetic markers except for 11q23, represented the focus of this study for clinical relevance of gene abnormalities and prognostic analysis. Because 11q23 rearrangements are mostly associated with *MLL* fusion genes and have been considered to bear similar clinical impact as *MLL*-PTD mutations,[34-36,44] which are not recognizable at karyotypic level, patients with these chromosomal changes were included into the series of 605 patients for prognostic analysis. Groups II and III consisted of, respectively, the 2 most common leukemia subtypes with translocation, namely *CBF* leukemias with *AML1* (*CBF*α)–*ETO* (158 cases) or *CBF*β-*MYH11* fusion (18 cases) and APL with *PML-RAR*α fusion (387 cases) or the rare variant *NPM1-RAR*α (1 case).[6,14,15] Although the prognostic value of these 2 groups has been established, these patients were included to overview the frequencies, distributions, and correlations of molecular mutations in the whole AML setting. In addition, there was a small group of 16 patients with relatively rare prognostic cytogenetic markers,[45] including t(3;3), t(9;22), −7, del(5q), del(7q), and complex translocation (for details see supplemental Table 1, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article).

This study was approved by the ethics committees of all participating centers. All patients were given informed consent for both treatment and cryopreservation of BM and peripheral blood according to the Declaration of Helsinki.

Treatment protocols

For APL (AML-M3) patients with t (15;17), all-*trans* retinoic acid and arsenic trioxide–based treatment was given for the induction and consolidation therapy.[14]

Other AML patients received standard first-line treatment in a DA-like regimen, which consisted of daunorubicin 45 mg/m^2 on days 1-3 and cytarabine 100-150 mg/m^2 on days 1-7. In the consolidation therapy, young patients were treated with high-dose cytarabine-based chemotherapy. Because of small number of the patients (n = 47) who received allogeneic stem cell transplantation, these patients were not separated out for further analysis. The chemotherapy consolidation for elderly patients was decided by the physicians in an individualized manner, as described previously.[46]

Cytogenetic and molecular genetic analysis

Cytogenetic and molecular studies were performed centrally at SIH and ZIH. The BM samples of de novo AML patients were studied mostly by R- and/or G-banding analysis, and were confirmed in most cases with relevant molecular markers. Chromosomal abnormalities were described according to the International System for Human Cytogenetic Nomenclature.[9]

Genomic DNA and total RNA were extracted as described previously.[40] Initially, we screened mutations in several genes, including *MDR1*, *BCL2*, *P53*, *XPA*, *ATM*, *SULT1C2*, *KIAA1244*, *COL7A1*, *N-RAS*, *NPM1*, and *IDH1* in some patients. Because sequence variations in the first 8 genes proved to be single nucleotide polymorphisms in 384 control samples from unrelated healthy individuals (data not shown), and because they were not included in current guidelines or practices of prognostic predicting,[8,45] no further analysis were performed on these genes. The remaining 3 genes revealed a certain percentage of mutations, and therefore our efforts were focused on these genes and on other previously known mutations such as *FLT3*-ITD and *FLT3*-TKD and *C-KIT*, *CEPBA*, *WT1*, *ASXL1*, *DNMT3A*, *MLL*, *IDH2*, and *TET2* gene mutations. Because the mutations of *FLT3*-TKD, *N-RAS*, *NPM1*, *IDH1*, and *IDH2* were clearly concentrated,[24,25,33] we used a chip-based matrix assisted laser desorption/ionization time-of-flight mass spectrometry (MALDI-TOF MS) analysis system (iPLEX; Sequenom) to perform high-throughput genotyping assays for analysis of the mutational status of these genes. For mutations of *FLT3*-ITD (in ITD region) and those in the *C-KIT*, *CEPBA*, *WT1*, *ASXL1*, *DNMT3A*, and *TET2* genes, samples were analyzed by whole-gene sequencing. In addition, a multiplex RT-PCR strategy was used to detect 6 *MLL*-related common fusion genes, including *MLL-AF9*, *MLL-AF10*, *MLL-AF6*, *MLL-ELL*, *MLL-ENL*, and *MLL-AF17*. Briefly, all samples were screened with 2 parallel multiplex RT-PCR reactions. If there were positive PCR fragments in the samples, split-out PCR was performed to determine the fusion gene type.[47] The mutational status of *MLL*-PTD and fusion genes such as *AML1* (*CBF*α)–*ETO*, *CBF*β-*MYH11*, and *PML-RAR*α were determined by RT-PCR.[22]

Quantitative real-time RT-PCR

After Turbo DNase (Ambion) treatment, 1 μg of total RNA was used for cDNA synthesis using the M-MLV First Strand Kit (Invitrogen). Real-time PCR was performed in ABI PRISM 7900HT using SYBR Premix Ex Taq (TaKaRa). The -fold change was calculated based on the $2^{-\Delta Ct} \times 1000$ method after normalization to the transcript level of the housekeeping gene *GAPDH*. Primer sequences used in the real-time RT-PCR are listed in supplemental Table 2.

Microarray expression profiling and methylation analysis

Affymetrix Human Genome U133 Plus 2.0 Array GeneChip microarrays were used to assess the total RNA samples (Affymetrix). DNA samples were extracted for the HG18 Methylation 2.1M Deluxe Promoter Array (NimbleGen) to identify the methylated DNA regions. The procedure and statistical analysis were performed as described previously.[40] All microarray data are available in the NCBI Gene Expression Omnibus (GEO) under accession number GSE27244.

Statistical analyses

For clinical analysis, complete remission (CR) was defined according to the criteria of the International Working Group.[48] The Fisher exact *P* test was used to compare differences in the CR rates. A 1-way ANOVA was used to compare the age, WBC count, and BM blasts at diagnosis in different groups. The relationship between different gene mutations was analyzed by

Table 1. Clinical characteristics of 1185 AML patients

Characteristics	AML without prognostic cytogenetic markers (group I)*	*CBF* leukemias (group II)	APL (AML-M3; group III)
Sex, no. of the patients (%)			
Male	348 (29.4)	104 (8.8)	199 (16.8)
Female	257 (21.7)	72 (6.1)	189 (15.9)
Mean age, y	43.2 ± 18.9	31.4 ± 19.5	34.7 ± 17.1
Range	18-86	18-75	18-80
No. of the patients aged < 60†	481	160	354
Median WBC count, × 10^9/L (range)	13.35 (0.5-453)	10.05 (0.8-177.9)	2.9 (0.3-205.7)
Median BM blasts, % (range)	69 (22.5-97)	60 (23.5-91)	64 (22.5-91.0)
FAB subtype, no. of the patients (%)			
M0	10 (0.8)		
M1	33 (2.8)		
M2a	120 (10.1)		
M2b		124 (10.5)	
M3			388 (32.7)
M4	188 (15.9)	25 (2.1)	
M4eo		18 (1.5)	
M5	200 (16.9)	5 (0.4)	
M6	23 (1.9)		
M7	2 (0.2)		
Not classified	29 (2.4)	4 (3.4)	

In addition to groups I, II, and III, there was a small group of 16 cases with relatively rare prognostic cytogenetic markers (see text).
Not classified refers to the AML patients without typical morphological characteristics and could not be defined by FAB classification.
WBC indicates white blood cell; BM, bone marrow; and no., number
*Except for those with 11q23 (see text).
†These patients were treated intensively in group I and II (see text).

Kendall's τ-b correlation coefficients. Overall survival (OS) was measured as the time from the date of disease diagnosis to death (failure) or alive at last follow-up (censored).[48] Event-free survival (EFS) was defined as the time from disease diagnosis to treatment failure such as relapse, refractory disease, death, or alive in CR at last follow-up (censored). Kaplan-Meier analysis was used to calculate the distribution of OS and EFS.[48] A log-rank comparison was performed to compare the differences in survival times. Binary logistic regression and the Cox model was used for the multivariate analysis of associations between mutational status and the achievement of CR and OS and of EFS, respectively.[49] A limited backward selection procedure was used to exclude redundant variates.[49] All of the above statistical procedures were performed with the SPSS Version 16.0 statistical software package.

Results

Patient characteristics

The characteristics of the 1185 de novo AML patients are summarized in Table 1. Particular attention for clinical analysis was given to 605 patients who lacked cytogenetic prognostic markers other than 11q23 abnormalities (group I, 51.1%). The relatively high number of patients with group III (APL, AML-M3) could have resulted from a preference for SIH by patients because of the successful all-*trans* retinoic acid and arsenic trioxide–based therapy. In addition, our data showed a relatively low incidence of M4eo after a careful examination of *CBFβ-MYH11* was performed in all morphologically M4 patients, which might reflect the difference in genetic backgrounds between the Chinese and white populations, in agreement with a previous large Chinese AML series.[50]

Frequencies and distribution of gene mutations

Among the 605 group I patients, *FLT3* mutations were found in 61 (10.8%), *C-KIT* in 30 (5.4%), *N-RAS* in 34 (5.9%), *NPM1* in 122 (20.9%), *CEBPA* in 123 (22.0%), *WT1* in 20 (3.7%), *ASXL1* in 27 (5.2%), *DNMT3A* in 73 (12.3%), *MLL* in 83 (14.0%), *IDH1* in 52 (9.3%), *IDH2* in 53 (9.8%), and *TET2* in 65 (12.7%); 452 (74.7%) patients were found to have at least one mutation. In group II patients with *CBF* leukemias, the most frequent mutations in addition to those caused by chromosomal translocations were *C-KIT* (25.6%) and *N-RAS* (9.7%), which differed from the situation in group III (APL, AML-M3), in whom mutations other than *PML-RARα* were mainly *FLT3* (13.4%) and *N-RAS* (5.4%) mutations (supplemental Figure 1).

As we recently reported,[40] a mutated *DNMT3A* gene was mostly associated with a myelomonocytic or monocytic morphology in the FAB classification ($P < .001$), with a frequency of 10.0% (22 of 219) and 18.2% (38 of 209) in M4 and M5, respectively. We identified 13 potential new types of *DNMT3A* mutations, which are shown in Figure 1A. All of these sequence variations were checked in a series of 384 control samples from unrelated healthy individuals and none was observed. We found that 3 M1, 1 M2 with *AML1* (*CBFα*)–*ETO* fusion, 1 M3 with *PML-RARα* fusion, 2 M6, and 2 M2 without *CBF* fusion patients also carried previously reported *DNMT3A* mutations.[37,40] The 2 patients with M6 presented monocytic features in nonerythroid lineages, whereas each of the M2 *CBF* and M3 patients experienced CNS involvement in spite of a standard intrathecal prophylaxis.

With regard to the class I gene mutations, *FLT3*-ITD and/or *FLT3*-TKD mutations exhibited an extremely lower incidence in *CBF* leukemias (2 of 176, 1.1%) than in other subtypes of AML ($P < .001$), whereas these were frequently present in addition to the *PML-RARα* fusion in the M3 subtype. Conversely, *C-KIT* mutations were most commonly seen in *CBF* leukemias (45 of 176, 25.6%, $P < .001$). In contrast, *N-RAS* mutations were distributed evenly in the different AML subtypes. Interestingly, among 259 patients bearing class I mutations, only 8 (3.1%) had overlapping of these markers, whereas all others carried mutation of only one gene.

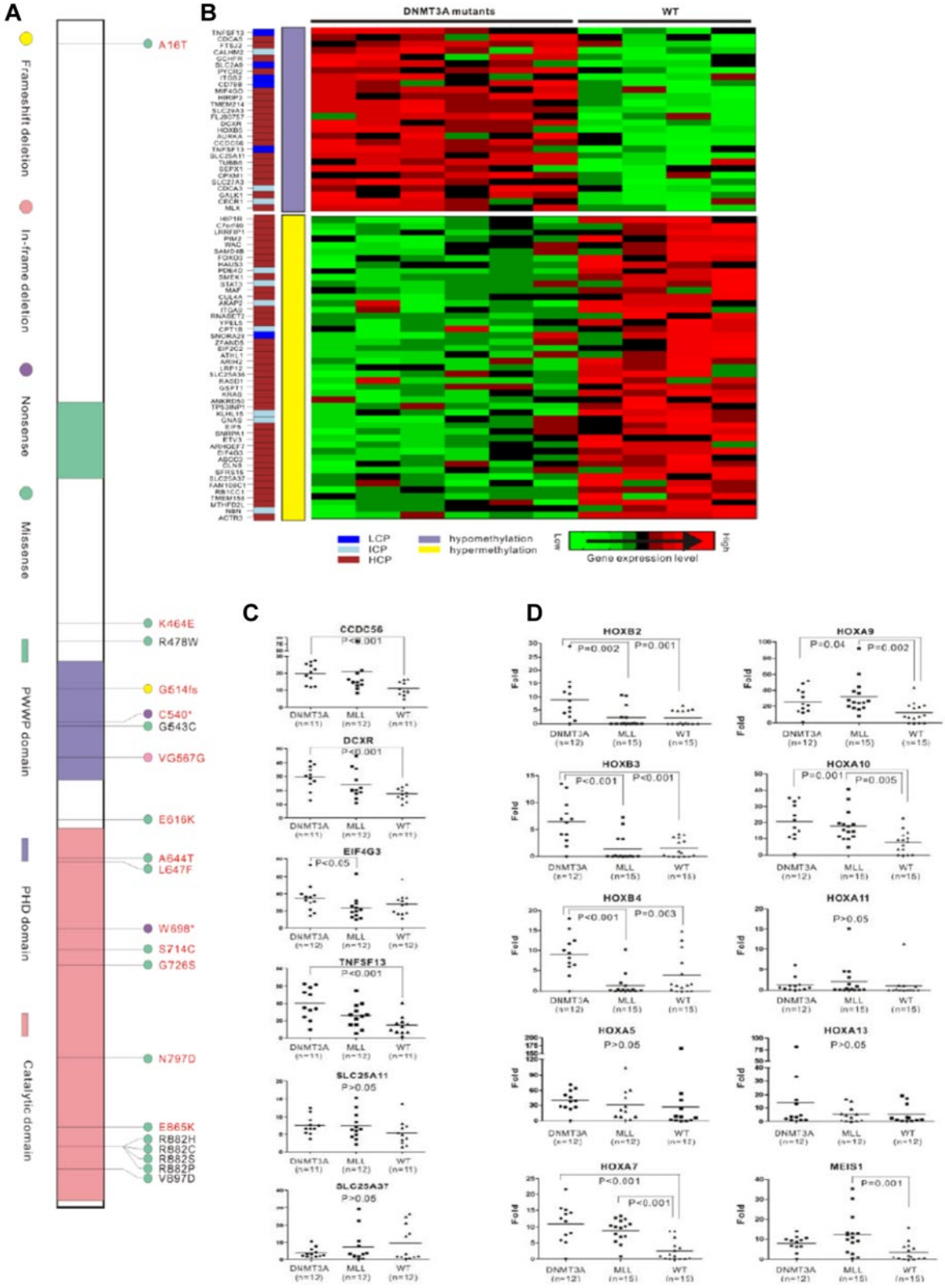

Figure 1. *DNMT3A* mutations in AML. (A) Three conserved domains in *DNMT3A* are shown: the PWWP domain, which targets the enzyme to nucleic acids; the cysteine-rich PHD zinc-finger domain, which interacts with unmodified histone H3; and the highly conserved catalytic domain in the C-terminal region. The mutations in AML previously reported by us are marked in black, and the newly detected mutations in the present study are in red. The most common missense mutations are predicted to affect amino acid R882. A total of 37 AML patients had the R882H mutation, 24 with R882C, 1 with R882S, and 1 with R882P in our 1178 samples. (B) Correlation analysis of gene expression and DNA methylation. The CpG content of the promoter sequences of the genes is color coded in a separate column (left lane), including low CpG content (LCP), intermediate CpG content (ICP), and high CpG content (HCP). Hypomethylation or hypermethylation in the middle lane indicates the CpG methylated level of genes in *DNMT3A*-mutated samples compared with samples without *DNMT3A* mutations. Cluster of differently expressed genes are shown on the right. Raw microarray data of gene expression and DNA methylation were published previously.[40] (C) Quantitative RT-PCR analysis of genes associated with hematopoiesis and epigenetics regulation that were up- or down-regulated and accompanied by DNA methylation changes in microarray analysis in patients with *DNMT3A* mutations (DNMT3A), *MLL* abnormalities (MLL), or without these 2 types of aberrations (WT). (D) Quantitative RT-PCR analysis of genes in distinct *HOX* families in patients with *DNMT3A* mutations (DNMT3A), *MLL* abnormalities (MLL), or without these 2 types of aberrations (WT).

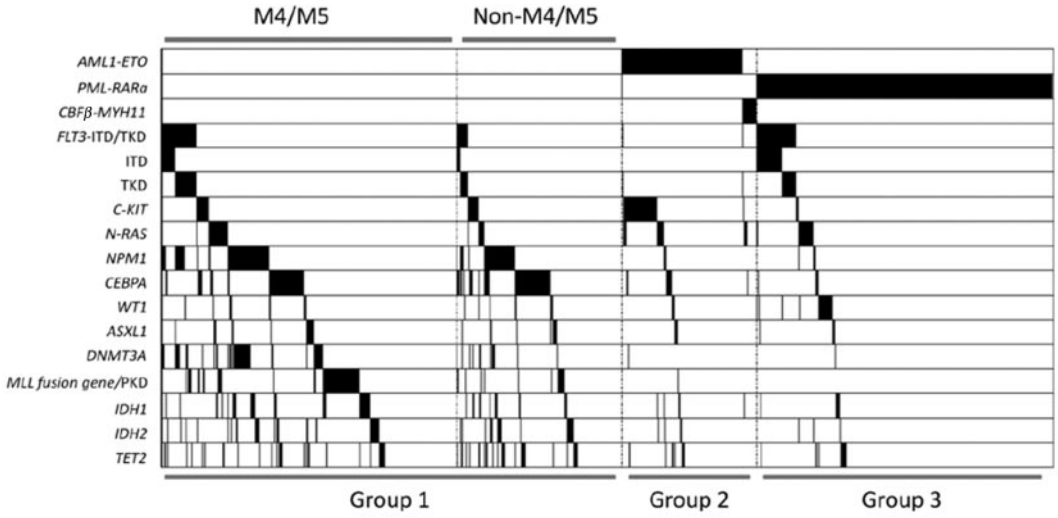

Figure 2. Mutation status of *AML1-ETO*, *PML-RARα*, *CBFβ-MYH11*, *FLT3*, *C-KIT*, *N-RAS*, *NPM1*, *CEBPA*, *WT1*, *ASXL1*, *DNMT3A*, *MLL*, *IDH1*, *IDH2*, and *TET2*. Black shadow indicates mutation cases.

Class II mutations involving *NPM1* and *CEBPA*, as well as mutants of genes related to epigenetic regulation such as *DNMT3A*, *MLL*, *IDH1*, *IDH2*, and *TET2*, tended to occur in AML patients without chromosome translocations (all $P < .001$). However, *ASXL1* ($P = .116$) and *WT1* ($P = .296$) mutations seemed to be distributed equally in cytogenetically normal or abnormal AML groups. Interestingly, in contrast to *DNMT3A* ($P = .002$) and *MLL* ($P = .004$) mutations, which were closely related to a M4 or M5 phenotype, *CEBPA* mutations favored a non-M4 and non-M5 phenotype ($P < .001$). Mutation of another epigenetic regulator, *TET2*, also showed this tendency ($P = .056$). The phenomenon of overlapping mutations in the same patient was quite frequent. Some mutations were even statistically correlated. *DNMT3A* (43 of 129, $P < .001$), *FLT3* (23 of129, $P < .001$), *IDH1* (17 of 129, $P = .046$), *IDH2* (26 of 129, $P < .001$), and *TET2* (16 of 129, $P = .030$) were significantly associated with *NPM1* mutations. Associations between *DNMT3A* and *FLT3*, *IDH1*, and *IDH2* were also observed ($P = .003$, .010, and .001, respectively). Among group I patients, frequent associations between the mutations of *NPM1* and *FLT3* ($P < .001$), *DNMT3A* ($P < .001$), *IDH1* ($P = .056$), and *IDH2* ($P < .001$), but not *TET2* ($P = .710$), were observed. Whereas mutations of *CEBPA* and *TET2* were highly correlated in all the patients ($P < .001$), only marginal correlation was identified in group I patients ($P = .053$). Notably, *MLL* abnormalities seldom coexisted with other mutations. Figure 2 shows the gene mutational status, including distributions and frequencies among distinct AML subtypes.

Correlation between gene-expression levels and DNA methylation status associated with *DNMT3A* mutations

We previously reported that aberrant DNA methyltransferase activity due to *DNMT3A* mutations could change DNA methylation and alter gene expression.[40] Interestingly, through in-depth exploration of the possible consequences of the *DNMT3A* mutations using microarray data from gene-expression profiling and whole-genomic DNA methylation, we identified a correlation between gene-expression levels and the DNA methylation status in some genes; 28 up-regulated genes with DNA hypomethylation and 47 down-regulated genes accompanied with DNA hypermethylation were revealed (Figure 1B). Among these, some genes were associated with hematopoiesis and epigenetics regulation, including *CCDC56*, *DCXR*, *TNFSF13*, and *SLC25A11* (up-regulated) and *SCL25A37* and *EIF4G3* (down-regulated). A correlation was revealed between the gene-expression levels of *CCDC56*, *DCXR*, and *TNFSF13* and the mutation of *DNMT3A*, whereas no up-regulation of these 3 genes was observed in patients with *MLL* rearrangement (Figure 1C).

It has been reported that increased expression of multiple homeobox (*HOX*) genes such as *HOXA7*, *HOXA9*, *HOXA10*, and *MEIS1* due to *MLL* abnormalities play an important role in leukemogenesis.[40] Recently, we demonstrated an up-regulation of *HOXB* family genes and the *IDH1* gene among patients with *DNMT3A* mutations.[40] We also observed from this study that although *MLL* and *DNMT3A* mutations seldom overlapped, they shared a common feature of poor prognosis in distinct patient populations. Therefore, we tried to address possible association of expression levels of different *HOX* genes with *MLL* or *DNMT3A* abnormalities. Interestingly, members of the *HOXB* family were found to be overexpressed only in a group with *DNMT3A* mutations, whereas up-regulation of the *MEIS1* gene could be observed only in patients with *MLL* abnormalities. Overexpression of *HOXA7*, *HOXA9*, and *HOXA10* genes was observed in both the *DNMT3A* and *MLL* mutation groups, contrarily to the situation of *HOXA5* and *HOXA13*, for which expression was not affected by *DNMT3A* and *MLL* abnormalities (Figure 1D). Although we previously found hypomethylation in genomic sequences of some *HOXB* family members, which might contribute to the overexpression status of these genes,[40] no obvious changes were found in *HOXA* family genes in the present study, so the exact molecular mechanisms for their up-regulation need further investigation.

Molecular markers and clinical aspects

Regarding possible association with clinical features, we found that mutations of *NPM1*, *CEBPA*, and nearly all of the genes regulating epigenetics (*DNMT3A*, *IDH1*, *IDH2*, and *TET2*) except for *MLL* rearrangements were associated with elderly age at diagnosis; *FLT3*, *NPM1*, *CEBPA*, *DNMT3A*, *MLL*, and *IDH2* mutations were associated with high WBC count at presentation; and *NPM1*, *FLT3*,

Table 2. Gene mutations and clinical aspects

Gene mutations	Sex: No. of the patients Male	Female	Mean age, y	Median WBC count, x10^9/L (range)	Median BM blasts, % (range)
***FLT3* ITD or TKD (missing = 54)**					
Mutated	68	47	38.2 ± 19.5	23.5 (0.5-447.6)	70.5 (22.5-94.0)
Unmutated	563	453	38.4 ± 18.8	7 (0.3-390.0)	65.0 (22.5-97.0)
P	.489		.903	< .001	< .001
***C-KIT* (missing = 63)**					
Mutated	47	31	35.3 ± 19.2	13.6 (0.8-197.0)	67.0 (37.6-90.0)
Unmutated	577	467	38.7 ± 18.9	7.7 (0.3-453.0)	65.0 (22.5-97.0)
P	.411		.127	.912	.453
***N-RAS* (missing = 59)**					
Mutated	46	28	38.5 ± 19.0	17.5 (1.0-185.7)	68.0 (37.0-97.0)
Unmutated	583	469	38.9 ± 20.2	7.6 (0.3-453.0)	65.0 (22.5-97.0)
P	.278		.854	.269	.139
***NPM1* (missing = 36)**					
Mutated	61	68	50.1 ± 16.1	29.3 (0.6-389.4)	71.5 (23.0-97.0)
Unmutated	576	444	37.1 ± 18.9	7.25 (0.3-453.0)	65.0 (22.5-97.0)
P	.049		< .001	< .001	< .001
***CEBPA* (missing = 54)**					
Mutated	84	54	43.2 ± 17.0	13.95 (1.0-453.0)	68.0 (25.5-91.0)
Unmutated	546	447	37.7 ± 19.2	7.1 (0.3-447.6)	65.0 (22.5-97.0)
P	.201		.001	< .001	.291
***WT1* (missing = 128)**					
Mutated	24	23	36.3 ± 18.9	10.2 (0.9-138.9)	69.0 (23.5-90.0)
Unmutated	565	445	38.6 ± 19.0	8.1 (0.3-453.0)	65.5 (22.5-97.0)
P	.550		.416	.528	.325
***ASXL1* (missing = 168)**					
Mutated	20	15	42.9 ± 21.5	11.8 (0.8-142.0)	62.0 (22.5-87.0)
Unmutated	550	432	38.4 ± 18.9	8.0 (0.3-453.0)	66.3 (22.5-97.0)
P	> .999		.162	.488	.096
***DNMT3A* (missing = 44)**					
Mutated	45	30	53.5 ± 15.5	37.9 (1.1-447.6)	78.0 (32.0-97.0)
Unmutated	592	474	37.5 ± 18.9	7.3 (0.3-453.0)	65.0 (22.5-97.0)
P	.548		< .001	< .001	< .001
***MLL* (missing = 20)**					
Abnormal	48	36	38.9 ± 18.6	10.95 (0.6-447.6)	74.0 (28.5-96.0)
Normal	601	480	38.5 ± 19.1	7.8 (0.3-453.0)	65.0 (28.5-97.0)
P	.820		.844	.003	< .001
***IDH1* (missing = 78)**					
Mutated	34	30	47.5 ± 18.1	10.1 (0.6-255.0)	68.0 (35.0-97.0)
Unmutated	585	458	37.7 ± 19.0	7.8 (0.3-453.0)	65.0 (22.5-96.0)
P	.698		< .001	.828	.872
***IDH2* (missing = 128)**					
Mutated	30	31	51.8 ± 15.9	12.1 (1.1-447.6)	68.0 (30.5-94.0)
Unmutated	558	438	37.8 ± 18.9	8.0 (0.3-453.0)	65.5 (22.5-97.0)
P	.353		< .001	.014	.566
***TET2* (missing = 175)**					
Mutated	40	46	45.9 ± 18.5	12.4 (0.9-453.0)	67.0 (23.5-95.0)
Unmutated	523	401	38.0 ± 18.8	7.9 (0.3-447.6)	65.5 (22.5-97.0)
P	.144		< .001	.224	.910

MLL variants include *MLL* fusion genes and PTD mutations. Missing refers to the samples failed to detect the mutation results in assays.
WBC indicates white blood cell; BM, bone marrow; and no., number.

DNMT3A, and *MLL* mutations were associated with a higher percentage of blasts in the BM (Table 2).

Response to induction therapy

In univariate analysis of the 605 patients in group I, *DNMT3A* mutations, *MLL* abnormalities, and *N-RAS* mutations were associated with a statistically significant lower CR rate (45.8%, 48.2%, and 41.2%, respectively; $P = .014$, .022, and .030, respectively) in contrast to *CEBPA* mutations (67.5%), which conferred a higher CR rate ($P = .030$). However, only biallelic (75.3%), but not monoallelic (56.0%), *CEBPA* mutations were associated with favorable response to the treatment ($P = .003$ and 1.000, respectively). Because *NPM1* mutations were frequently associated with abnormalities of *DNMT3A*, *FLT3*, *IDH1*, and *IDH2*, we performed further analysis using the combination of *NPM1* mutations with each of the above-mentioned mutations to investigate their

Table 3. Multivariate analysis for clinical and molecular variables of CR, OS, and EFS

	CR		OS		EFS	
Variables	***P***	**OR (95% CI)**	***P***	**OR (95% CI)**	***P***	**OR (95% CI)**
Age	< .001	0.976 (0.965-0.987)	< .001	1.018 (1.011-1.027)	< .001	1.013 (1.006-1.020)
WBC count	.020	0.996 (0.992-0.999)	.022	1.002 (1.000-1.004)	.044	1.002 (1.000-1.004)
BM blasts	NS		NS		NS	
FLT3 ITD or TKD	NS		NS		NS	
C-KIT	NS		NS		NS	
N-RAS	NS		NS		NS	
NPM1 m+/DNMT3A m-	.001	2.533 (1.430-4.488)	.014	0.626 (0.431-0.910)	.012	0.638 (0.450-0.906)
Bi-allelic *CEBPA*	.005	2.450 (1.319-4.553)	< .001	0.396 (0.214-0.650)	.001	0.488 (0.319-0.746)
WT1	NS		NS		NS	
ASXL1	NS		NS		NS	
DNMT3A	.036	0.486 (0.248-0.953)	.005	1.753 (1.189-2.583)	.010	1.638 (1.123-2.388)
MLL variants	NS		.002	1.803 (1.240-2.623)	.004	1.642 (1.167-2.311)
IDH1	NS		NS		NS	
IDH2	NS		NS		NS	
TET2	NS		NS		NS	

MLL variants include *MLL* fusion genes and PTD mutations.

potential prognostic impact. Obviously, *DNMT3A* mutations could separate the patients with *NPM1* mutations into 2 distinct prognostic groups: a subpopulation of *NPM1* mutations without *DNMT3A* mutations (*NPM1*m^+/*DNMT3A*m^-) was related to a significantly higher CR rate (72.3%, $P = .017$); however, mutations of *IDH1* ($P = .265$) and *IDH2* ($P = .218$), and *FLT3*-ITD ($P = .164$) or *FLT3*-TKD ($P = .318$) did not add more prognostic value among patients with the *NPM1* mutation (supplemental Table 3). In M4 and M5 patients, similar results were achieved ($P = .056$ for *DNMT3A* mutations and $P = .742$, .736, .661, and .737 for *IDH1*, *IDH2*, *FLT3*-ITD, and *FLT3*-TKD mutations, respectively).When 89 cases without gene mutations were grouped, they seemed to have an intermediate prognosis in terms of CR (64.0%).

A complete list of covariates that entered the multivariate model is provided in Table 3. Multivariate analysis of group I patients indicated that *NPM1*m^+/*DNMT3A*m^- and biallelic *CEBPA* mutations (*CEBPA*m^+) were independent factors associated with favorable CR rate, and *DNMT3A* mutations (*DNMT3A*m^+) were associated with a lower CR rate. Two other independent clinical factors, WBC count and age, were also unfavorable for CR rate. In the age-adjusted population (n = 481) who were younger than 60 years and treated intensively, *MLL* abnormalities, *TET2* mutations, and BM blasts proportion predicted unfavorable CR rate independently (Table 4).

Survival analysis

Among 605 group I patients, the median OS and EFS were 15.0 ± 1.9 and 8.0 ± 1.2 months, respectively. In univariate analysis, *DNMT3A* mutations and *MLL* abnormalities suggested a poor prognosis (both $P < .001$ for OS; and $P = .001$ and $P < .001$ for EFS, respectively). Although *N-RAS* mutation cases showed an inferior EFS ($P = .016$), OS was only marginally affected ($P = .084$). Favorable OS and EFS were achieved in the *CEBPA*m^+ patients ($P = .002$ and .005 for OS and EFS, respectively). Further analysis showed that only biallelic *CEBPA*m^+ status was associated with better treatment outcome (both $P < .001$ for OS and EFS, respectively). There was no statistical significance in OS and EFS in *FLT3*, *C-KIT*, *WT1*, *ASXL1*, *IDH1*, *IDH2*, and *TET2* mutations ($P = .169$ and .371, .317 and .165, .815 and .587, .765 and .717, .257 and .731, .339 and .770, .148 and .074, respectively). *NPM1* mutation did not predict OS ($P = .409$) and EFS ($P = .274$). However, in patients with *NPM1* mutations, it was the mutation of *DNMT3A* ($P < .001$ and $P = .002$ for OS and EFS, respectively), but not of *FLT3* (ITD: $P = .231$ and .156, for OS and EFS, respectively; TKD: $P = .314$ and .204, for OS and EFS, respectively), *IDH1* ($P = .457$ and .552 for OS and EFS, respectively) or *IDH2* ($P = .863$ and .843 for OS and EFS, respectively) that

Table 4. Multivariate analysis for clinical and molecular variables of CR, OS, and EFS in younger patients

	CR		OS		EFS	
Variables	***P***	**OR (95% CI)**	***P***	**OR (95% CI)**	***P***	**OR (95% CI)**
WBC count	NS		NS		NS	
BM blasts	0.011	0.983 (0.970-0.996)	NS		NS	
FLT3 ITD or TKD	NS		NS		NS	
C-KIT	NS		NS		NS	
N-RAS	NS		NS		NS	
NPM1 m+/DNMT3A m-	NS		.035	0.598 (0.370-0.964)	.060	0.659 (0.426-1.018)
Bi-allelic *CEBPA*	NS		.001	0.365 (0.204-0.656)	.001	0.442 (0.271-0.722)
WT1	NS		NS		NS	
ASXL1	NS		NS		NS	
DNMT3A	NS		< .001	2.637 (1.600-4.347)	.002	2.149 (1.329-3.473)
MLL variants	.021	0.487 (0.264-0.897)	.013	1.671 (1.116-2.501)	.015	1.576 (1.093-2.273)
IDH1	NS		NS		NS	
IDH2	NS		NS		NS	
TET2	.069	0.520 (0.257-1.053)	NS		NS	

MLL variants include *MLL* fusion genes and PTD mutations.

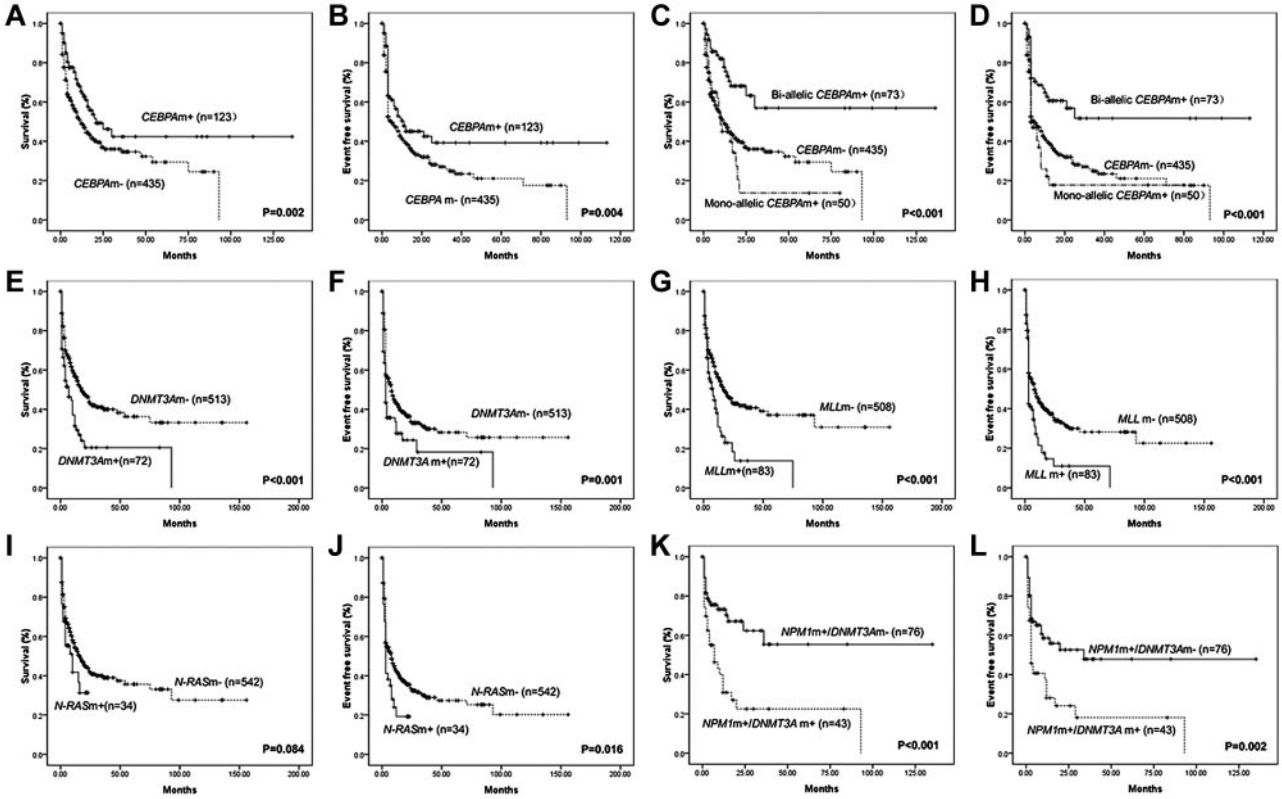

Figure 3. Kaplan-Meier curves for OS and EFS according to genotypes with statistical significance in univariate analysis. (A-B) The median OS and EFS of patients with or without *CEBPA* mutations (*CEBPA*m$^+$ or *CEBPA*m$^-$) were 21.0 ± 5.8 months (mo) and 12.0 ± 1.5 mo ($P = .002$), 11.0 ± 5.1 mo, and 5.0 ± 0.5 mo ($P = .004$), respectively. (C-D) The median OS and EFS of patients with biallelic or monoallelic *CEBPA* mutations were not reached (NR) and 10.0 ± 1.6 mo ($P < .001$), and NR and 3.0 ± 0.7 mo ($P = .001$), compared with wild-type *CEBPA* patients. (E-F) The median OS and EFS of patients with or without *DNMT3A* mutations (*DNMT3A*m$^+$ or *DNMT3A*m$^-$) were 7.0 ± 2.1 mo and 18.0 ± 2.3 mo ($P < .001$), 3.0 ± 0.3 mo and 8.0 ± 1.2 mo ($P = .001$), respectively. (G-H) The median OS and EFS of patients with or without *MLL* abnormalities (*MLL*m$^+$ or *MLL*m$^-$) were 8.0 ± 2.2 mo and 17.0 ± 2.4 mo ($P < .001$), 3.0 ± 0.2 mo and 8.0 ± 1.4 mo ($P < .001$), respectively. (I-J) The median OS and EFS of patients with or without *N-RAS* mutations (*N-RAS*m$^+$ or *N-RAS*m$^-$) were 10.0 ± 4.2 mo and 17.0 ± 2.1 mo ($P = .084$), 3.0 ± 0.3 mo and 8.0 ± 1.3 mo ($P = .006$), respectively. (K-L) The median OS and EFS of patients with *NPM1* mutation but no *DNMT3A* mutation (*NPM1*m$^+$/*DNMT3A*m$^-$) were NR and 34.0 mo, whereas *NPM1* mutation cases with *DNMT3A* mutations (*NPM1*m$^+$/*DNMT3A*m$^+$) had inferior OS (7.0 ± 3.4 mo, $P < .001$) and EFS (3.0 ± 0.6 mo, $P = .002$).

helped to discriminate the 2 prognostic groups. Figure 3 shows the Kaplan-Meier curves for OS and EFS according to genotypes with statistical significance in univariate analysis. In M4 and M5 patients, only *DNMT3A* mutations ($P = .001$ and .006 for OS and EFS, respectively) could subdivide the patients with *NPM1* mutations. The existence of the mutation of *IDH1* ($P = .585$ and .941 for OS and EFS, respectively), *IDH2* ($P = .275$ and .118 for OS and EFS, respectively), *FLT3*-ITD ($P = .807$ and .507 for OS and EFS, respectively), and TKD ($P = .988$ and .621, respectively) could not help to further stratify the *NPM1*m$^+$ patients.

In multivariate analysis, *DNMT3A*m$^+$ and *MLL* rearrangements (*MLL*m$^+$) were independent factors predicting poor prognosis; biallelic *CEBPA*m$^+$ and *NPM1*m$^+$/*DNMT3A*m$^-$ conferred a better OS and EFS (Table 3). Age and WBC count were also independent factors related to prognosis. Among younger patients who received standard induction and consolidation, the results were similar to the entire group, and the mutational status of the above-mentioned 4 genes still bore prognostic significance, whereas WBC count no longer predicted OS and EFS.

Using molecular markers that had proved to be significantly related to prognosis in multivariate analysis, we could stratify AML patients without cytogenetic markers into 3 prognostic groups: (1) a favorable-risk group with biallelic *CEBPA*m$^+$ or *NPM1*m$^+$/*DNMT3A*m$^-$ status; (2) a poor-risk group with *DNMT3A*m$^+$ or *MLL*m$^+$ status; and (3) an intermediate group with all of the other remaining cases (Figure 4). The prognosis for the 89 patients without detectable gene mutations also corresponded to an intermediate status in terms of both OS and EFS (supplemental Figure 3). Therefore, according to the gene mutational status, the 605 group I patients could be clearly classified into distinct prognostic subgroups.

Discussion

It has long been appreciated that cytogenetic factors are independent predictors for the prognosis of AML patients.[7,8,10,15,45] However, for > 50% of AML patients, no cytogenetic markers can be found. Genetic mutations that escape cytogenetic detection have increasingly been discovered, and these mutations may serve as potential markers to extend the prognostic parameters in AML. Enormous efforts have been made to clarify the correlation between molecular changes and the clinical outcome of AML patients, allowing further dissection of AML into molecular subtypes with distinctive prognosis and therapy responses.[16,37,45] Nevertheless, the clinical value of some genetic mutations remain controversial, and the frequency and prognostic impact of some newly discovered mutations have not yet been well documented. A systematic investigation of genetic mutations in large series of patients is essential to determine their clinical relevance.

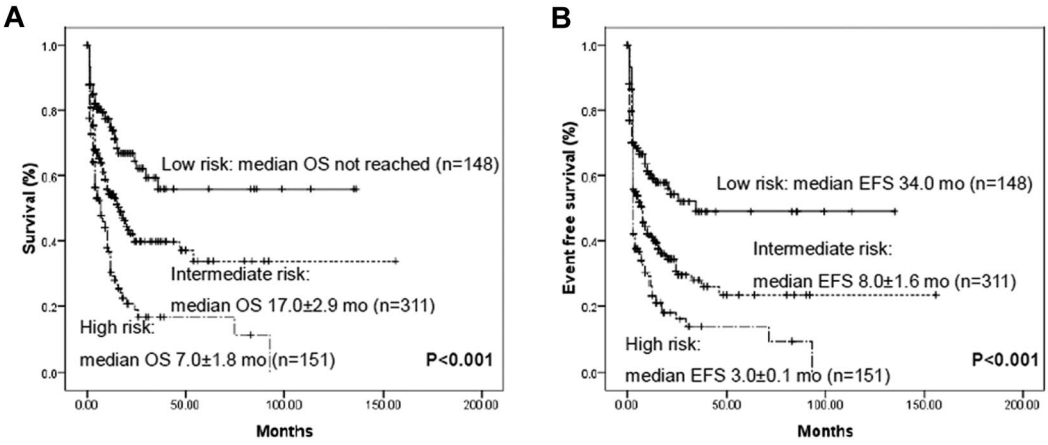

Figure 4. Kaplan-Meier curves for OS and EFS according to genotypes with statistical significance in multivariate analysis. All AML patients without cytogenetic prognostic markers could be divided into 3 prognostic groups using 4 marker combinations: low- risk, biallelic *CEBPA*m$^+$ and/or *NPM1*m$^+$/*DNMT3A*m$^-$; high-risk, *DNMT3A*m$^+$ and/or *MLL*m$^+$; and intermediate, all remaining cases. Very few patients were repeatedly calculated in each group because of the concurrence of different mutations.

In this study, we attempted to clarify the value of a cluster of molecular markers other than cytogenetic factors in the stratification of AML patients into different prognostic groups. One important finding was that the recently reported *DNMT3A* mutations indeed had a higher incidence in M4 and M5 subtypes (10.0% and 18.2%, respectively, $P < .001$) in a much larger patient cohort in this study.[40] The fact that *DNMT3A* mutations were also identified in 2 cases of M6 with erythromonocytic leukemia provides further evidence that *DNMT3A* mutations are restricted to the monocytic lineage involvement in AML. We also detected, for the first time, *DNMT3A* mutations in one M2 patient carrying *AML1*(*CBF*α)-*ETO* and in one M3 patient with *PML-RAR*α. These 2 patients quickly developed CNS leukemia even under intrathecal prophylaxis, which is reminiscent of the characteristic extramedullary involvement in monocytic leukemia. In addition, in support of the report by Ley[37] and extending our previous results,[40] *DNMT3A* mutations were highly associated with a very poor prognosis in AML patients.

Combining the mutations discovered by Ley's study and the potential sequence variations identified in this series, an interesting situation emerged: the sequence changes occurring in the PHD domain of the DNMT3A protein were microdeletions or nonsense mutations resulting in loss-of-function of the protein (except for a homozygous G543C mutation with abnormal interaction to histone H3 that we reported previously[40]). In contrast, the sequence changes found in the catalytic domain consisted mostly of missense alterations, leading to reduction of enzymatic activity according to biochemistry assay.[40] DNA methylation is a crucial epigenetic modification of the genome that is involved in many cellular processes, including the regulation of gene expression and structural remodeling of chromatin.[30-32] In the present study, we further analyzed the aberrant DNA methylation status due to *DNMT3A* mutations in relationship to gene-expression patterns in AML patients. Directly or indirectly due to abnormal DNA methyltransferase activity, these deregulated genes might further contribute to the pathogenesis of leukemia. We found that whereas overexpression of *HOXB* genes could be detected only in the *DNMT3A*-mutated group and *MEIS1* was found only in *MLL* abnormal patients, up-regulation of *HOXA7*, *HOXA9*, and *HOXA10* genes was found in both *DNMT3A* mutated and *MLL* abnormal patients. The leukemogenesis of *DNMT3A* mutations and *MLL* aberration might share some common pathways, albeit with different characteristics. Some other important genes associated with hematopoiesis and epigenetics regulation, such as *CCDC56*, *DCXR*, and *TNFSF13*, were also affected by *DNMT3A* mutation, which deserves mechanistic study with regard to leukemogenesis. Considering the poor prognosis of patients with *DNMT3A* mutations, together with the biologic data,[40] we propose *DNMT3A* mutation as a "driver" mutation that plays an essential role in the pathogenesis of leukemia involving the monocytic lineage.

We found that leukemia samples from 452 of 605 cases (74.7%) in group I contained at least one of the mutations. It has been generally accepted that 2 classes of gene mutations cooperate in AML pathogenesis. Class I mutations such as those of *C-KIT*, *FLT3*, and *N-RAS* are associated with activated signal transduction and provide a proliferative and survival advantage to the hematopoietic progenitors.[16] However, they often show subtype-restricted distribution in AML, as evidenced by a rather specific *C-KIT* mutation as the second hit in the pathogenesis of *CBF* leukemias[18] and the unique high incidence of *FLT3* mutations in APL (AML-M3),[14] although *N-RAS* mutations displayed an even distribution across all major subtypes of AML. The class II gene mutations affecting transcription regulation and causing impaired differentiation often overlapped with other molecular defects, and coexistence patterns of some mutations were recognizable. Nevertheless, *MLL* mutations seemed to be mutually exclusive events, seldom overlapping with other mutations. Interestingly, in our preliminary morphology analysis, *CEBPA*, *MLL*, and *DNMT3A* seemed to be correlated with distinct morphologic phenotypes within the AML-M4 subtype: patients with *DNMT3A* mutations and *MLL* variants tended to have a major monocytic lineage involvement in BM, in contrast to those with *CEBPA* mutations, who had more blasts of granulocytic lineage. This might be explained by the respective roles of *CEBPA* in myeloid differentiation and of *MLL* and *DNMT3A* in the control of differentiation/growth of monocytic cells. Our results suggest a necessity of cooperation between distinct genetic events in leukemogenesis, although further investigation of the underlying mechanism is warranted.

It is worth pointing out that abnormalities of epigenetic regulation seem to play an essential role in the pathogenesis of AML, as evidenced by the fact that *DNMT3A* is a DNA methyltransferase whereas *MLL* is a histone methyltransferase. In addition, *IDH1*, *IDH2* and *TET2* mutations were found to result in DNA hypermethylation,[31-33,51] and a group of histone methyltransferase genes (eg, *MLL2*, *UTX*, and *SETD2*) have recently been found to be mutated in hematologic malignancies. Mutations of *EVI1*, the

protein product of which is assumed to interact with DNMT3A and DNMT3B, was recently reported to be associated with poor prognosis in AML.[1] Conversely, mutations of *EZH2*, encoding a histone methyltransferase that could interact with DNMT3A, were described in lymphomas and myelodysplastic syndromes.[52,53] Taking these data into consideration, we propose that gene mutations involved in epigenetic regulation may be considered as a third class, apart from class I and II mutations, because they not only belong to a distinct regulatory network, but also might share common features of aggressive disease, poor prognosis, and older age onset (with the exception of *MLL* abnormalities).

In the present study, logistic-regression analyses showed that *DNMT3A* mutations represented independent, unfavorable prognostic factors for remission induction with conventional daunorubicin and cytarabine-based chemotherapy. In contrast, biallelic *CEBPA* or *NPM1* mutations without *DNMT3A* mutations were associated with a favorable response. Age and WBC count also proved to be independent factors of adverse outcome. However, in a relatively young patient population, only *MLL* and *TET2* abnormalities were independently associated with unfavorable induction results. Cox regression analysis revealed that *DNMT3A* mutations and *MLL* rearrangements were associated with an inferior OS and EFS, whereas biallelic *CEBPA* mutations or *NPM1* mutations without *DNMT3A* changes independently predicted favorable OS and EFS. Again, these 4 genes were proved to be the only independent prognostic factors in relatively young patients in predicting OS and EFS. *NPM1* mutations with wild-type *FLT3*-ITD was shown to be an important favorable genotype in AML patients without cytogenetic changes in previous studies.[20-22] Our series failed to show the difference between *NPM1*m^+/*FLT3*-ITDm^+ and *NPM1*m^+/*FLT3*-ITDm^- groups, possibly due to the lower frequency of *FLT3*-ITD mutations in our series in cytogenetically normal patients (23 of 605, 3.8%), which might hamper further stratification of *NPM1*m^+ patients and decrease the sensitivity of the statistics.

In summary, we have established a new stratification system with which to subclassify the prognosis of AML without cytogenetic prognostic factors according to the mutation patterns of 4 genes. Evaluation of molecular markers in AML, especially through detection of *DNMT3A*, *MLL*, *NPM1*, and *CEBPA* mutations, can therefore be recommended. Further studies will be focused on using different treatment strategies according to AML genotypes. For example, enhanced therapies, such as high-dose anthracyclines and DNA-methylation–regulatory agents in elderly patients with *DNMT3A* mutations, might be evaluated for their potential to improve clinical outcome in AML.

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Authorship

Contribution: S.-J.C, J.J., and Z.C. were the principal investigators who conceived the study; S.-J.C., J.J., Z.C., and Y.S. coordinated and oversaw the study; Y.S., Y.-M.Z., X.F., Q.-R.W., and J.-Y.S. performed most of the experiments; Z.-H.G. and X.-J.Y. were responsible for bioinformatics investigation; Q.-R.W. and J.-Y.S. participated in the validation experiments; C.-L.J and H.Y. contributed in sample treating and PCR amplification; Y.S., X.F., F.-F.C., Y.-Y.W, B.C., and H.-M.C. gathered detailed clinical information for the study and helped to perform the clinical analysis; Y.-M.Z. and J.-Y.S. participated in the PCR assay and Sequenom analysis; and Z.C., S.-J.C., and Y.S. wrote the manuscript.

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