Mutant *DNMT3A:* a marker of poor prognosis in acute myeloid leukemia

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The prevalence, the prognostic effect, and interaction with other molecular markers of *DNMT3A* mutations was studied in 415 patients with acute myeloid leukemia (AML) younger than 60 years. We show mutations in *DNMT3A* in 96 of 415 patients with newly diagnosed AML (23.1%). Univariate Cox regression analysis showed that patients with *DNMT3A*^{mutant} AML show significantly worse overall survival (OS; *P* = .022; hazard ratio [HR], 1.38; 95% confidence interval [CI], 1.04-1.81), and relapse-free survival (RFS; *P* = .005; HR, 1.52; 95% CI, 1.13-2.05) than *DNMT3A*$^{wild\text{-}type}$ AMLs. In a multivariable analysis, *DNMT3A* mutations express independent unfavorable prognostic value for OS (*P* = .003; HR, 1.82; 95% CI, 1.2-2.7) and RFS (*P* < .001; HR, 2.2; 95% CI, 1.4-3.3). In a composite genotypic subset of cytogenetic intermediate-risk AML without *FLT3-ITD* and *NPM1* mutations, this association is particularly evident (OS: *P* = .013; HR, 2.09; 95% CI, 1.16-3.77; RFS: *P* = .001; HR, 2.65; 95% CI, 1.48-4.89). The effect of *DNMT3A* mutations in human AML remains elusive, because *DNMT3A*^{mutant} AMLs did not express a methylation or gene expression signature that discriminates them from patients with *DNMT3A*$^{wild\text{-}type}$ AML. We conclude that *DNMT3A* mutation status is an important factor to consider for risk stratification of patients with AML. (*Blood*. 2012;119(24):5824-5831)

Introduction

Acute myeloid leukemia (AML) is a heterogeneous group of malignancies with different clinical behavior and different responses to therapy. AML can be classified according to distinct cytogenetic and genetic abnormalities, as well as on epigenetic differences that have been shown to be invaluable to guide risk assessment and choice of treatment.[1-3] Although insight into the disease has improved in past years, the discovery and validation of new discriminative biomarkers remain of utmost value to improve outcome prediction, in particular for those patients who cannot be classified yet with the currently available biomarkers.

The effect of the association between DNA methylation and various types of human cancer has become a main research domain over the past decade. In conjunction with other epigenetic processes such as histone modifications and interfering RNA, DNA methylation represents an essential component of the transcriptional regulation machinery[4] and of the repressive chromatin structure.[5] According to their structure in mammals, 3 families of DNA methyltransferase enzymes have been identified.[6,7] They are related to each other and probably diverged early in eukaryotic evolution.[7] Mammalian DNA methyltransferases (DNMTs) catalyze the transfer of a methyl group onto the 5′-position of cytosine at CpG dinucleotides.[8,9] DNMT3A and DNMT3B catalyze de novo DNA methylation, whereas DNMT1 is primarily responsible for maintenance methylation.[4,7,10-12]

Recently, a somatic change in *DNMT3A* was identified in human AML, resulting in Arg-to-His substitution at codon R882, located in the methyltransferase domain, causing loss of methylation activity.[13] Recurrent *DNMT3A* mutations at multiple sites, including codon R882, were subsequently detected in a large cohort of patients with AML.[14-16] *DNMT3A* mutations were reported to frequently occur in AML with a normal karyotype and associated with French-American-British (FAB) M5 morphology.[14-16] An association with unique DNA methylation and gene expression profiles[16] and unfavorable prognosis were suggested.[15]

In this study we investigated the distribution of *DNMT3A* mutations in a cohort of 415 AML cases. The relation with cytogenetic and molecular risk categories and the effect of *DNMT3A* mutations on treatment outcome were analyzed, and the association of *DNMT3A* mutations with gene methylation and gene expression patterns in AML was investigated. We provide evidence that the *DNMT3A* mutation status in AML is an important factor to consider for risk stratification of the disease.

Methods

Patients and patient samples and mutation analysis

BM aspirates of patients with AML were collected after written informed consent in accordance with the Declaration of Helsinki. All experiments

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described were approved by the Erasmus University Medical Center Institutional Review Board. Patients with AML were treated according to the HOVON (Dutch-Belgian Hematology-Oncology Cooperative Group) AML protocols HO04, HO04A, HO29, HO42, HO42A, and HO43 (http://www.hovon.nl). Molecular aberrations in *FLT3* (internal tandem duplication [ITD] or tyrosine kinase domain), *NPM1*, *NRAS*, *KRAS*, *IDH1-2, KIT, WT1*, and *CEBPA* and *EVI1* overexpression were determined as described previously[17-21]

DNMT3A mutation analysis

It was recently reported that most mutations in *DNMT3A* occurred in the second part of the gene.[14-16] Therefore, *DNMT3A* mutations were determined in the present study by cDNA amplifications with the use of FWA, 5′-ACGACAGCGATGAGAGTGAC-3′, and REVA, 5′-CCCAATCACCAGATCGAATG-3′, or FWC, 5′-TGAGGACTCCATCACGGTG-3′, and REVC, 5′-CGGTATTTCCGCCTCTGTG-3′. All PCRs were performed in the presence of 25mM deoxynucleoside triphosphate, 20 pmol primers, 1mM $MgCl_2$, Taq polymerase, DMSO, and 10 times buffer (Invitrogen). Cycling conditions were as follows: 1 cycle for 5 minutes at 94°C, 35 cycles for 1 minute at 94°C, 1 minute at 56°C, 1 minute at 72°C, and 1 cycle for 10 minutes at 72°C. All PCR products were subsequently sequenced with the appropriate primers (FWA, 5′-ACGACAGCGATGAGAGTGAC-3′, and FWB, 5′-GCTTTCTGGAGTGTGCGTAC-3′, or FWC, 5′-TGAGGACTCCATCACGGTG-3′, and FWD, 5′-TGATTGATGCCAAAGAAGTGTC-3′) applying an ABI-PRISM3100 genetic analyzer (Applied Biosystems). *DNMT3A* mutations are considered new somatic mutations in cases in which they were not reported as single nucleotide polymorphism (SNP) according to dbSNP version 132. *DNMT3A* mutations in AML around the hotspot site (R882) were confirmed by cDNA amplifications with FWD, 5′-TGATTGATGCCAAAGAAGTGTC-3′, and REVE, 5′-GACTGGCACGCTCCATGAC-3′, followed by denaturing HPLC analyses with the use of a Transgenomic WAVE system. Products were run at 60.4°C.

Gene expression profiling and methylation profiling

All AML cases were previously profiled for gene expression[22] (Gene Expression Omnibus; National Center for Biotechnology Information; www.ncbi.nlm.nih.gov/geo; accession no. GSE6891) and profiled for methylation[1] (accession no. GSE18700). For gene expression–based or gene methylation–based classification of *DNMT3A* in different defined intermediate risk subtypes, gene expression profiling and DNA methylation profiling (HpaII tiny fragment enrichment by ligation-mediated PCR) data of the HOVON cohort were used to derive predictive signatures with the use of a logistic regression model with Lasso regularization as reported previously.[23]

Statistics and survival analyses

The relation between *DNMT3A* mutations and various patient characteristics was determined by the chi square test or Fisher exact test for the categorical variables and by the Mann-Whitney *U* test for continuous variables. We distinguished the following cytogenetic risk categories: (1) favorable: t(8;21) or inv(16); (2) adverse: inv(3)/t(3,3), t(6;9), 11q23 abnormalities other than t(9;11), del5, del5(q), del7, del7(q), or t(9;22) and monosomal karyotypes[24]; and (3) intermediate risk: the remaining AML cases. Overall survival (OS) end points were death (failure) and alive at last follow-up (censored), as measured from entry onto trial. Relapse-free survival (RFS) end points were disease relapse or death from any cause, measured from complete remission (CR) onto trial. Estimates of the survival distribution for OS and RFS were calculated by the Kaplan-Meier method. Cox proportional hazards model was used in both univariate and multivariate analyses, in which the reported *P* values correspond to the Wald test. Andersen-Gill model was used to analyze survival outcome in relation to a time-dependent covariate.

Results

DNMT3A is frequently mutated in AML

We performed mutation analysis of the second part of *DNMT3A*, encompassing the PHD and methyltransferase domains, in 415 patients with newly diagnosed AML. This part of the gene, which has been reported to harbor > 95% of the mutations found in *DNMT3A*, represents exons 11 until the last exon, that is, exon 23. We found mutations in 96 of the 415 patients (23.1%), that is, 58 missense mutations at position R882 and 38 mutations at other positions (supplemental Figure 1 and supplemental Table 1, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). In 2 AML cases 2 *DNMT3A* mutations were found simultaneously (supplemental Table 1). All *DNMT3A* mutations were considered somatic according to dbSNP (version 132). Patients with AML with *DNMT3A* mutations presented with significantly higher age, higher white blood cell (WBC) counts, and higher platelet counts compared with $DNMT3A^{\text{wild-type}}$ AMLs (Table 1). In line with previous reports, 37.5% of FAB M5 AMLs (36 of 100) carried mutations in *DNMT3A*. Seventy-two of 96 $DNMT3A^{\text{mutant}}$ AMLs (75%) carried a normal karyotype (NK-AML). AMLs with t(8;21), inv(16), inv(3;3)/t(3,3), and trisomy 21 never carried *DNMT3A* mutations. Furthermore, *DNMT3A* mutations were rare in complex karyotype AMLs (Table 1). We also separately analyzed 25 patients with acute promyelocytic leukemia (APL), that is, with a t(15;17), and found only 1 patient with a *DNMT3A* mutation (R882; supplemental Table 1).

DNMT3A mutations significantly associated with ITDs in FLT3 ($FLT3^{\text{ITD}}$; $P = .002$) as well as with mutations in *NPM1* ($P < .001$) or *IDH1* ($P < .001$). In contrast, *DNMT3A* mutations were significantly underrepresented in *EVI1*-overexpressing AMLs. The presence of *DNMT3A* mutations did not correlate with recurrent mutations in *CEBPA, NRAS, KRAS, WT1, FLT3* (TKD), or *KIT* (Table 1).

The frequency of mutations in *DNMT3A* in patients with AML > 60 years, which were analyzed separately, was 10 of 63 (16%; supplemental Table 1). Four of 16 AMLs with myelodysplastic syndrome–related changes carried *DNMT3A* mutations. Mutations in *DNMT3A* were also found in 2 of 11 therapy-related AML cases (supplemental Table 1).

$DNMT3A^{\text{mutant}}$ is an independent prognostic marker in AML

The median follow-up of the 415 patients with AML that remained alive was 115.7 months (range, 7.2-224.1 months). The median OS of patients with $DNMT3A^{\text{mutant}}$ AML was shorter than that of patients with $DNMT3A^{\text{wild-type}}$ AML (11.9 months vs 24.0 months). $DNMT3A^{\text{mutant}}$ AMLs did not show a significantly different CR rate compared with patients with $DNMT3A^{\text{wild-type}}$ AML (ie, 88.54% for $DNMT3A^{\text{mutant}}$ vs 79.3% for $DNMT3A^{\text{wild-type}}$ AML; $P = .058$). The univariate Cox regression analysis showed that the presence of *DNMT3A* mutation is an unfavorable prognostic factor for OS ($P = .022$; hazard ratio [HR], 1.38; 95% confidence interval [CI],1.04-1.81) and RFS ($P = .005$; HR, 1.52; 95% CI, 1.13-2.05) among all patients with AML (Figure 1A-B).

Multivariate analysis showed that $DNMT3A^{\text{mutant}}$ is an independent prognostic indicator for unfavorable OS ($P = .003$; HR, 1.82; 95% CI, 1.2-2.7) and RFS ($P < .001$; HR, 2.2; 94% CI, 1.4-3.3) in the entire patient group when we consider age (continuous variable), WBC count, cytogenetic risk, $NPM1^{\text{mutant}}$, $FLT3^{\text{ITD}}$,

Table 1. Clinical characteristics of the 415 patients with AML

	DNMT3A$^{wild\text{-}type}$ (n = 319)	*DNMT3A*^{mutant} (n = 96)	*DNMT3A* R882 mutation (n = 58)	*DNMT3A*^{mutant} except for R882 codon (n = 38)	*P*
Sex, male, n (%)	163 (51.10)	47 (48.96)	31 (53.45)	16 (42.11)	.8
Median age at study entry, y (range)	41 (15-60)	50.5 (18-60)	51 (18-60)	51 (18-60)	< .001*
Median WBC count at diagnosis, × 10^9/L (range)	23.05 (0.6-274)	52.9 (1.1-278)	54.85 (2-278)	48 (1.1-220)	< .001*
Median platelet count, × 10^9/L (range)	50.5 (3-998)	64 (10-494)	64.5 (10-494)	63.5 (10-267)	.003*
Median bone marrow blast, % (range)	68 (0-98)	68 (9-98)	70 (9-97)	65.5 (22-98)	.94*
FAB classification					.054†
M0	15 (4.7)	1 (1.0)	1 (1.7)	0	.18†
M1	72 (22.6)	15 (15.6)	9 (15.5)	6 (15.8)	.27†
M2	81 (25.4)	23 (24.0)	11 (19.0)	12 (31.6)	.86†
M4	64 (20.1)	15 (15.6)	10 (17.2)	5 (13.2)	.58†
M5	64 (20.1)	36 (37.5)	23 (39.7)	13 (34.2)	< .001†
M6	5 (1.6)	1 (1.0)	0	1 (2.6)	‡
RAEB	13 (4.0)	3 (3.1)	2 (3.4)	1 (2.6)	.99†
ND	5 (1.6)	2 (2.1)	2 (3.4)	0	‡
Cytogenetics					
Normal karyotype	122 (38.2)	72 (75.0)	46 (79.3)	26 (68.4)	< .001†
t(8;21)	35 (11.9)	0	0	0	‡
inv(16)	34 (10.6)	0	0	0	‡
del5/del5(q)	13 (4.0)	2 (2.0)	1 (1.7)	1 (2.6)	.65
del7/del7q(q)	24 (7.5)	3 (3.1)	2 (3.4)	1 (2.6)	.78
inv(3;3)/t(3;3)	5 (1.5)	0	0	0	‡
Trisomy 8	27 (8.4)	5 (5.2)	3 (5.1)	2 (5.2)	.39
Trisomy 21	7 (2.1)	0	0	0	.86
11q23	25 (7.8)	1 (1.0)	0	1 (2.7)	.01
Monosomy	22 (6.9)	2 (2.0)	1 (1.7)	1 (2.7)	.08
Complex	34 (10.6)	4 (4.1)	3 (5.1)	1 (2.7)	.07
Cytogenetic risk group					< .001†
Favorable	57 (17.9)	0	0	0	
Intermediate	191 (59.9)	85 (88.5)	51 (88.0)	34 (89.4)	
Adverse	64 (20.0)	6 (6.3)	4 (6.9)	2 (5.3)	
ND	7 (2.2)	5 (5.2)	3 (5.1)	2 (5.3)	
Molecular abnormalities					
FLT3^{ITD}	77 (24.1)	39 (40.6)	24 (41.3)	15 (39.4)	.002
FLT3^{TKD}	26 (8.2)	15 (15.6)	9 (15.5)	6 (15.7)	.052
NPM1 mutation	60 (18.8)	73 (76.0)	46 (79.3)	27 (71.0)	< .001
CEBPA^{SM}	7 (2.1)	1 (1.0)	1 (1.7)	0	.69†
CBPA^{DM}	21 (6.5)	2 (2.0)	0	2 (5.2)	.15
IDH1 mutation	11 (3.4)	22 (22.9)	18 (31.0)	4 (10.5)	< .001
IDH2 mutation	23 (7.2)	13 (13.5)	4 (6.9)	9 (23.6)	.086
cKit mutation	22 (6.9)	1 (1.0)	1 (1.7)	0	‡
WT1 mutation	21 (6.5)	3 (3.1)	1 (1.7)	2 (5.2)	.311
KRAS mutation	1 (0.3)	3 (3.1)	2 (3.4)	1 (2.6)	‡
NRAS mutation	34 (10.6)	8 (8.3)	4 (6.9)	4 (10.5)	.57
EVI1 overexpression	39 (12.2)	1 (1.0)	1 (1.7)	0	‡

P values not otherwise indicated were calculated with the 2-sided χ^2 test and are for the comparisons between no *DNMT3A* mutations and any *DNMT3A* mutation. Cytogenetic risk group favorable includes t(8;21), inv(16) or t(15;17); adverse, inv(3)/t(3;3), t(6;9), 11q23 abnormalities other than t(9;11), del5, del5(q), del7, del7(q), t(9;22) or monosomal karyotypes (MK); and intermediate, the remaining AML cases.

WBC indicates white blood cell; FAB, French-American-British; RAEB, refractory anemia with excess blasts; and ND, not determined.

**P* values were calculated with Wilcoxon (Mann-Whitney) test for the comparisons between no *DNMT3A* mutations and any *DNMT3A* mutation.

†*P* values were calculated with Fisher exact test for the comparisons between no *DNMT3A* mutations and any *DNMT3A* mutation.

‡Numbers were too low for reliable statistical analysis.

CEBPA double mutation (*CEBPA*^{DM}), *NRAS*^{mutant}, *IDH1*^{mutant}, *IDH2*^{mutant}, *WT1*^{mutant}, *cKIT*^{mutant}, and *EVI1* overexpression (Table 1). The variables that independently associated with OS were *DNMT3A*^{mutant}, *NPM1*^{mutant}, *FLT3*^{ITD}, *CEBPA*^{DM}, cytogenetic risk, and WBC count. Of these factors, *FLT3*^{ITD} and WBC count were not independently associated with RFS (Table 2). *DNMT3A*^{mutant} showed no independent prognostic value for CR in multivariate analyses.

To investigate whether allogeneic transplantation modifies the prognostic effect of *DNMT3A*^{mutant}, we applied Cox proportional hazards model with *DNMT3A* and allogeneic transplantation as a time-dependent covariate, including also an interaction of the 2. For overall survival, we observed a HR of 1.3507 ($P = .066$; 95% CI, 0.98-1.86) for *DNMT3A*^{mutant}. The interaction term with allogeneic transplantation was not significant ($P = .615$; HR, 1.173; 95% CI, 0.623-2.189).

When we analyzed *DNMT3A* mutations at position R882 only, we observed an association with inferior outcome as well, that is, OS ($P = .018$; HR, 1.49; 95% CI, 1.07-2.07) and RFS ($P = .029$; HR, 1.50; 95% CI, 1.04-2.15) in this series of patients with AML (supplemental Figure 2A-B).

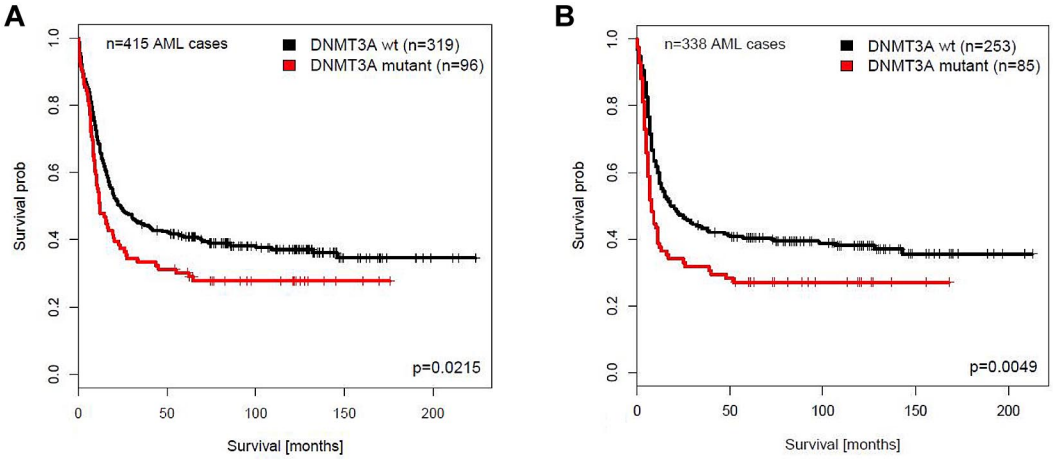

Figure 1. Survival analyses of patients with AML with or without *DNMT3A* mutations. Survival analyses were performed on the AML patient cohort < 60 years of age and from which the APLs were excluded. (A) Overall survival (OS). (B) Relapse-free survival (RFS).

DNMT3A^{mutant} predicts poor treatment response in *NPM1*$^{wild\text{-}type}$/*FLT3*$^{wild\text{-}type}$/*CEBPA*$^{wild\text{-}type}$ AML

Mutations in *DNMT3A* particularly were associated with molecular abnormalities, for example, mutations *NPM1*, *FLT3* (*FLT3*^{ITD}), *IDH1*, or *IDH2* (Table 1). *DNMT3A* mutations were not preferentially found in *CEBPA*^{DM} AMLs (Table 1). We studied the predictive value of *DNMT3A* mutations in the distinct molecularly defined AML subcategories. Subgroup analysis found that in the AML cohort from which *CEBPA*^{DM} cases were excluded, *DNMT3A* mutations still predicted inferior OS ($P = .046$; HR, 1.325; 95% CI, 1.01-1.75) or RFS ($P = .023$; HR, 1.414; 95% CI, 1.05-1.91; Table 3; Figure 2A; supplemental Figure 3A). We next assessed the prognostic value of *DNMT3A* mutations in each of the following composite subgroups: *FLT3*^{ITD}/*NPM1*^{mutant}, *FLT3*$^{wild\text{-}type}$/*NPM1*^{mutant}, *FLT3*^{ITD}/*NPM1*$^{wild\text{-}type}$, or *FLT3*$^{wild\text{-}type}$/*NPM1*$^{wild\text{-}type}$. A significant inferior OS ($P = .002$; HR, 2.167; 95% CI, 1.32-3.56) and RFS ($P < .001$; HR, 2.781; 95% CI, 1.63-4.75) for *DNMT3A*^{mutant} AMLs was observed in the *NPM1*$^{wild\text{-}type}$/*FLT3*$^{wild\text{-}type}$ (no *FLT3*^{ITD}) subset (Figure 2B; supplemental Figure 3B), whereas *DNMT3A* mutations had no predictive value in the *NPM1*^{mutant}/*FLT3*$^{wild\text{-}type}$ and the *NPM1*^{mutant}/*FLT*^{ITD} subgroups (Table 3). The predictive value for inferior OS ($P = .002$; HR, 2.268; 95% CI, 1.36-3.79) and RFS ($P = .002$; HR, 2.503; 95% CI, 1.42-4.42) was also evident in the *NPM1*$^{wild\text{-}type}$/*FLT3*$^{wild\text{-}type}$/*CEBPA*$^{wild\text{-}type}$ (no *CEBPA*^{DM}) subset (Figure 2C; supplemental Figure 3C).

Because *DNMT3A* mutations were most frequently found in intermediate-risk AMLs (Table 1), we performed the same survival analyses in subgroups within this particular subset, with comparable results (Table 3). In the *FLT3*$^{wild\text{-}type}$/*NPM1*$^{wild\text{-}type}$ intermediate-risk AMLs, *DNMT3A* mutations were significantly associated with an inferior OS ($P = .013$; HR, 2.09; 95% CI, 1.16-3.77) and RFS ($P = .001$; HR, 2.70; 95% CI, 1.48-4.89; Figure 2E; supplemental Figure 3E). A significant predictive value of *DNMT3A* mutations for poor treatment response was also found for intermediate-risk *NPM1*$^{wild\text{-}type}$/*FLT3*$^{wild\text{-}type}$/*CEBPA*$^{wild\text{-}type}$ (no *CEBPA*^{DM}) AMLs (OS: $P = .017$; HR, 2.11; 95% CI, 1.145-3.907; RFS: $P = .016$; HR, 2.18; 95% CI, 1.153-4.125; Figure 2F; supplemental Figure 3F). We recently reported that *IDH1* mutations have prognostic value in *FLT3*$^{wild\text{-}type}$/*NPM1*$^{wild\text{-}type}$ as well.[21] Although the numbers were too low for a reliable statistical analysis, the high hazard ratios (Table 3) favor the hypothesis that the predictive value of *DNMT3A* mutations is independent of the presence of *IDH1* and *IDH2* mutations as well.

Table 2. Multivariable analysis of *DNMT3A* as a prognostic marker for OS and RFS

	OS		RFS	
	HR (95% CI)	*P*	HR (95% CI)	*P*
DNMT3A	1.82 (1.2-2.7)	.003	2.20 (1.4-3.3)	< .001
FLT3^{ITD}	1.67 (1.2-2.3)	.002	1.40 (0.9-2.0)	.060
NPM1^{mut}	0.44 (0.2-0.6)	< .001	0.40 (0.2-0.6)	< .001
CEBPA^{DM}	0.34 (0.1-0.7)	.005	0.40 (0.2-0.8)	.017
NRAS^{mut}	0.78 (0.4-1.3)	.341	0.70 (0.3-1.3)	.310
IDH1^{mut}	0.76 (0.4-1.3)	.330	0.87 (0.5-1.5)	.630
IDH2^{mut}	0.69 (0.4-1.1)	.170	0.70 (0.4-1.2)	.220
EVI1	1.43 (0.8-2.3)	.145	1.59 (0.8-2.8)	.120
WT1^{mut}	1.23 (0.7-2.1)	.430	1.40 (0.8-2.6)	.180
cKit^{mut}	0.54 (0.2-1.3)	.190	1.22 (0.5-2.8)	.630
Cytogenetic risk group*	1.77 (0.9-3.2)	.050	2.70 (1.3-5.4)	.003
Age	1.01 (0.9-1.0)	.125	1.001 (0.9-1.0)	.970
WBC count	1.002 (1.001-1.003)	.002	1.001 (0.9-1.0)	.140

Survival analysis was performed on young patients with newly diagnosed young AML (< 60 years) from which APL cases were excluded (n = 415). Age and WBC count were analyzed as continuous variables in the multivariate analysis.

OS indicates overall survival; RFS, relapse-free survival; HR, hazard ratio; CI, confidence interval; and WBC, white blood cell.

*Cytogenetic risk group favorable includes t(8;21), inv(16), or t(16;16); adverse, inv(3)/t(3;3), t(6;9), 11q23 abnormalities other than t(9;11), del5, del5(q), del7, del7(q), t(9;22), or monosomal karyotypes (MK); and intermediate, the remaining AML cases.

Table 3. *DNMT3A* as a prognostic marker for OS and RFS in distinct subsets of patients with AML

	OS			RFS		
	*DNMT3A**	HR	*P*	*DNMT3A**	HR	*P*
Subset type in total cohort†						
CEBPA^{wt}	94/392	1.325	.046	83/316	1.414	.023
FLT3^{wt} *NPM1*^{mut}	37/64	1.967	.075	34/58	1.912	.089
FLT3^{ITD} *NPM1*^{mut}	36/69	1.491	.179	32/61	1.616	.133
FLT3^{wt} *NPM1*^{wt}	20/235	2.167	.002	17/193	2.781	< .001
FLT3^{wt} *NPM1*^{wt} *IDH1*^{wt} *IDH2*^{wt}	10/203	2.830	.003	8/167	3.812‡	
FLT3^{wt} *NPM1*^{wt} *CBPA*^{wt}	18/215	2.268	.002	15/174	2.503	.002
FLT3^{wt} *NPM1*^{wt} *CBPA*^{wt} *IDH1*^{wt} *IDH2*^{wt}	8/184	3.987‡		6/149	3.783‡	
Normal karyotype	72/194	1.255	.216	67/164	1.524	.033
Intermediate risk group	85/276	1.330	.073	79/236	1.530	.011
Subset type in intermediate risk group§						
CEBPA^{wt}	83/254	1.243	.179	77/215	1.373	.064
FLT3^{wt} *NPM1*^{mut}	34/59	2.044	.074	31/54	2.108	.063
FLT3^{ITD} *NPM1*^{mut}	33/65	1.429	.249	31/59	1.673	.117
FLT3^{wt} *NPM1*^{wt}	16/117	2.097	.013	15/102	2.703	.001
FLT3^{wt} *NPM1*^{wt} *IDH1*^{wt} *IDH2*^{wt}	7/91	2.593‡		7/79	4.690‡	
FLT3^{wt} *NPM1*^{wt} *CBPA*^{wt}	14/98	2.114	.017	13/84	2.181	.016
FLT3^{wt} *NPM1*^{wt} *CBPA*^{wt} *IDH1*^{wt} *IDH2*^{wt}	5/73	4.711‡		5/62	4.616‡	

The overall survival (OS) and relapse-free survival (RFS) were determined in the distinct subgroups indicated.
HR indicates hazard ratio; *CEBPA*^{wt}, no *CEBPA*^{DM}; and *FLT3*^{wt}, no *FLT3*^{ITD}.
**DNMT3A* means the number of mutants detected among the total number of patients that were present in the indicated subgroup.
†Subgroups were defined within the total cohort of 415 patients < 60 years of age with AMLs and excluding the APLs.
‡Numbers were too low for reliable statistical analysis.
§Patients < 60 years with cytogenetically intermediate risk were studied.

DNMT3A mutations are enriched in a methylation and gene expression cluster of *NPM1*^{mutant}/*FLT3*^{ITD} AMLs

We investigated whether *DNMT3A*^{mutant} AMLs were associated with unique DNA methylation[25] or gene expression signatures.[22] Although *DNMT3A*^{mutant} AMLs did not express a exclusive methylation signature, as determined with the HELP assay[25] (Figure 3), methylation cluster 13 was enriched for *DNMT3A*^{mutant} AMLs (71.1% [n = 32 of 45] *DNMT3A*^{mutant} AML; Figure 3). Importantly, this cluster mainly contains FAB M4/M5 *NPM1*^{mutant}/*FLT3*^{ITD} AMLs.[25] This group of AMLs also showed a unique gene expression signature (supplemental Figure 4). In fact, patient samples within this cluster overexpress multiple *HOX* genes, a signature that is particularly associated with *NPM1*^{mutant} AMLs.[20,26,27] It has been proposed that the increased levels of HOX genes in these leukemias are caused by loss of methylation as the result of mutations in *DNMT3A*[16]. However, we observed no difference in *HOX* gene expression, for example, *HOXB5* expression levels between *NPM1*^{mutant}/*DNMT3A*^{mutant} and *NPM1*^{mutant}/*DNMT3A*$^{wild\text{-}type}$ AMLs (supplemental Figure 5). Thus, *HOXB5* overexpression, representative for the expression of multiple *HOX* genes in human AML, does not associate specifically with the presence of *DNMT3A* mutations.

Supervised analysis with the use of a logistic regression model with Lasso regularization was performed to compare the gene expression as well as the gene methylation signatures of *DNMT3A*^{mutant} versus *DNMT3A*$^{wild\text{-}type}$ AMLs in distinct subgroups, that is, in *NPM1*^{mutant}, *FLT3*^{ITD}, *NPM1*^{mutant}/*FLT3*^{ITD}, *NPM1* $^{wild\text{-}type}$/*FLT3*^{ITD}, *NPM1* ^{mutant}/*FLT3*$^{wild\text{-}type}$, and *NPM1*$^{wild\text{-}type}$/*FLT3*$^{wild\text{-}type}$ AMLs. We did not find a strong predictive signature for each dataset in any of the aforementioned AML subtypes within the intermediate risk group. Therefore, the role of mutated *DNMT3A* on DNA methylation and gene expression in human AMLs remains elusive.

Discussion

In this study, we identified mutations in *DNMT3A* in 96 of 415 newly diagnosed AMLs (23.1%). Of the 96 *DNMT3A* mutant AMLs, 85 belonged to the group of AMLs with an intermediate-risk cytogenetic profile. In fact, mutations in *DNMT3A* are as frequent in this group of AMLs as other common molecular abnormalities, such as mutations in *FLT3* (*FLT3*^{ITD}), *NPM1, IDH1*, or *IDH2*. As a matter of fact, *DNMT3A* mutations frequently are associated with these particular mutations, which is in strong concordance with data reported previously.[14-16]

Note that, with the use of th approach we chose to determine mutations in *DNMT3A*, a small number of *DNMT3A*^{mutant} AMLs may have been missed. We performed mutation analysis on cDNA in the second half of the *DNMT3A* gene only, that is, the region containing the PDH and methyltransferase domains and which, according to previous studies, harbors > 95% of the *DNMT3A* mutations.[14-16] Missense or frameshift mutations, leading to nonsense-mediated RNA decay, which represented only a minority of the mutations in previous studies, may have been missed as well because we used cDNA for nucleotide sequencing. However, the mutation rate we found is comparable with frequencies reported by others, suggesting that we identified most *DNMT3A* mutations in our cohort of AMLs and that the conclusions drawn in our study are valid.

Patients with *DNMT3A*^{mutant} have significantly worse OS than patients with *DNMT3A*$^{wild\text{-}type}$, independent of a number of *NPM1*, *FLT3*, or *CEBPA* mutations, and regardless of age, cytogenetic risk, and WBC count. Interestingly, by univariate analyses we show that in a composite genotypic subset of cytogenetic intermediate-risk AML without *FLT3*^{ITDs} and *NPM1* mutations this association was particularly evident (OS, *P* = .013; RFS, *P* = .001). A negative prognostic effect of *DNMT3A* mutations on AML with a NK and with wild-type *NPM1* and wild-type *FLT3* was previously reported by Thol et al,[15] emphasizing the relevance of this finding. Similar to

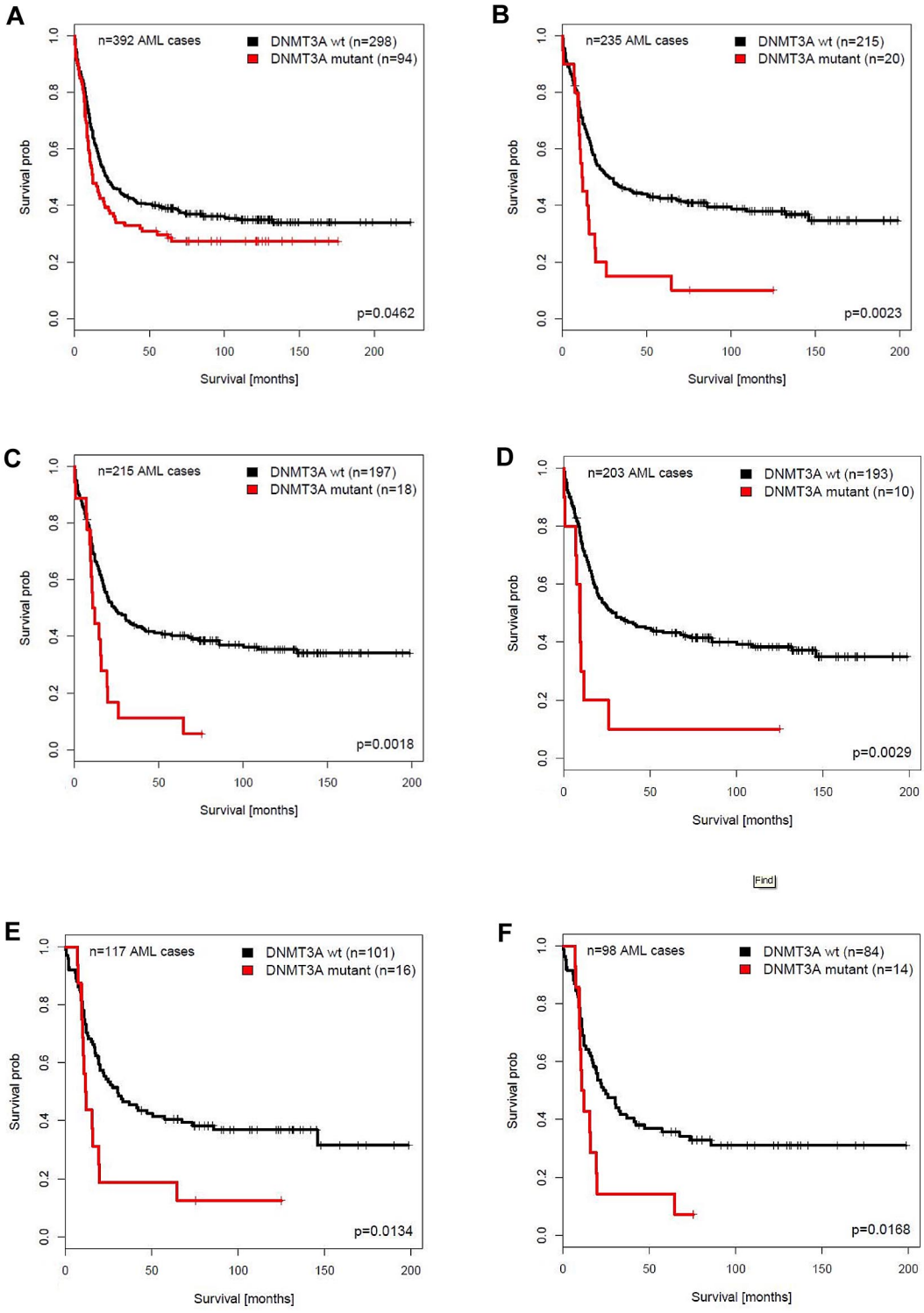

Figure 2. Survival analysis of patients with AML with or without *DNMT3A* mutations in distinct AML subgroups. Survival analyses were performed on the AML patient cohort < 60 years of age and from which the APLs were excluded. (A) OS of patients with AML, excluding patients with *CEBPA*^{DM} (n = 392). (B) OS of patients with AML without *FLT3*^{ITD} and without *NPM1* mutations (*FLT3*$^{wild\text{-}type}$/*NPM1*$^{wild\text{-}type}$; n = 235). (C) OS of patients with AML without *FLT3*^{ITD}, *NPM1* mutations, and *CEBPA*^{DM} (*FLT3*$^{wild\text{-}type}$/*NPM1*$^{wild\text{-}type}$/*CEBPA*$^{wild\text{-}type}$; n = 215). (D) OS of patients with AML without *FLT3*^{ITD}, *NPM1, IDH1,* and *IDH2* mutations (*FLT3*$^{wild\text{-}type}$/*NPM1*$^{wild\text{-}type}$/*IDH1*$^{wild\text{-}type}$/*IDH2*$^{wild\text{-}type}$; n = 203). (E) OS of patients with cytogenetically intermediate-risk AML without *FLT3*^{ITD} and without *NPM1* mutations (*FLT3*$^{wild\text{-}type}$/*NPM1*$^{wild\text{-}type}$; n = 117). (F) OS of patients with cytogenetically intermediate-risk AML without *FLT3*^{ITD}, *NPM1* mutations, and *CEBPA*^{DM} (*FLT3*$^{wild\text{-}type}$/*NPM1*$^{wild\text{-}type}$/*CEBPA*$^{wild\text{-}type}$; n = 98).

the report by Thol et al,[15] no effect of *DNMT3A* mutations on survival was found in the *FLT3*$^{wild\text{-}type}$/*NPM1*^{mutant} NK-AML subgroup. In contrast, the same investigators reported a significant worse OS for NK-AMLs with *DNMT3A*^{mutant}/*FLT3*^{ITD}/*NPM1*^{mutant} compared with *DNMT3A*^{widtype}/*FLT3*^{ITD}/*NPM1*^{mutant}, which was not evident in our study. Even though we studied the effects *DNMT3A* mutations on intermediate-risk AMLs, the discrepancy is remarkable and emphasizes the requirement of studies in larger cohorts of AML.

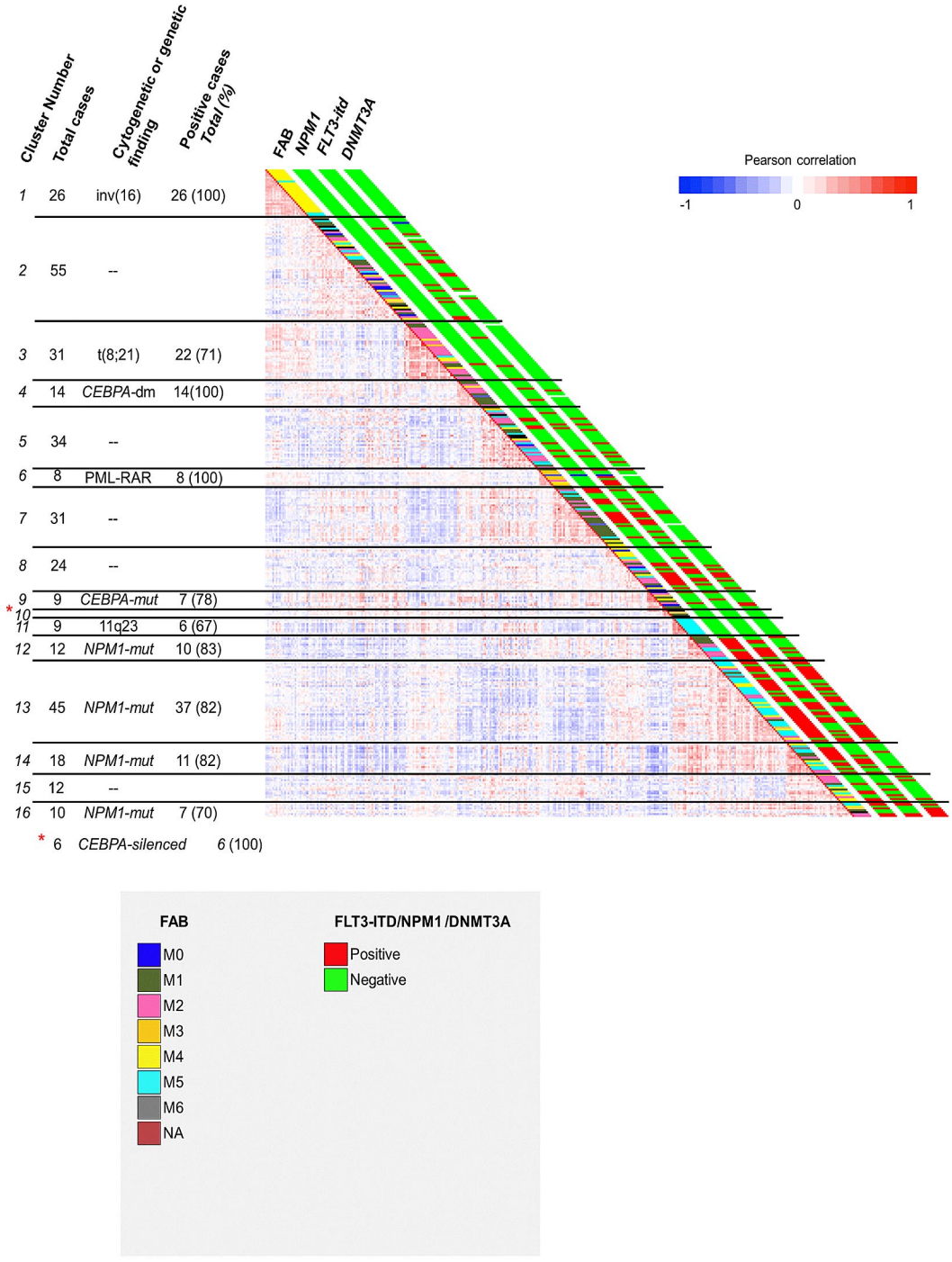

Figure 3. Clustering of AML patient samples according to methylation profiling with HELP. Distribution of *DNMT3A*^{mutant} and *DNMT3A*$^{wild\text{-}type}$ AMLs. Pearson correlation view based on methylation profiling of 334 AML cases (see previous figure from Figueroa et al[25]). The presence (red) or absence (green) of *NPM1* mutation, *FLT3*-ITD, or *DNMT3A* mutation is indicated for the individual AML cases adjacent to the correlation matrix. The distinct FAB types are indicated in different colors.

Mutations in *DNMT3A* would predict that those AML cases show a distinct DNA methylation pattern than cases without mutations. Yan et al reported that *DNMT3A* mutations caused loss of methylase activity, and they proposed that these alterations resulted in hypomethylation and uncontrolled expression of multiple *HOX* genes.[16] Although we showed that *DNMT3A*^{mutant} AMLs did not show a strong methylation signature, one methylation cluster (cluster 13) showed enrichment of *DNMT3A*^{mutant} cases (Figure 3).[25] Importantly, this cluster had already been reported to mainly consist of FAB M4/M5 AMLs with *NPM1*^{mutant}/*FLT3*^{ITD}.[1,20] Moreover, this group of patients also showed a unique gene expression signature, including overexpression of multiple *HOX* genes.[20,26,27] Therefore, the question should be raised whether this *HOX* gene signature, that is, the strong increase of expression of multiple *HOX* genes, is indeed caused by hypomethylation of the *HOX* gene promoters as the result of mutations in the *DNMT3A* gene, as was reported by Yan et al.[16] Multiple investigators have previously reported that the *HOX* gene signature is particularly associated with *NPM1*^{mutant} AMLs.[20,26,27] Our study found that *DNMT3A*^{mutant} AMLs without *NPM1* mutations did not present this

HOX gene signature; that is, *HOX* genes were not overexpressed in those AMLs (supplemental Figure 5). Furthermore, *HOX* gene overexpression was also found in *DNMT3A*$^{\text{wild-type}}$/*NPM1*$^{\text{mutant}}$ AMLs.[20,26,27] Importantly, Vassiliou et al[28] reported recently that expression of mutant *NPM1* in the myeloid compartment, using a *NPM1*-mutant knock-in mouse model, was associated with a strong up-regulation of *HOX* genes. Therefore, the role for mutated *DNMT3A* in aberrant control of *HOX* genes remains unclear. *DNMT3A*$^{\text{mutant}}$/*NPM1*$^{\text{mutant}}$ compound mice should further clarify what the effect of mutant *DNMT3A* is in myeloid progenitors and how it may affect *HOX* gene expression in the context of *NPM1* mutations.

Currently, patient age, cytogenetics, and mutations in *CEBPA, FLT3*, and *NPM1* at diagnosis are main prognostic factors in AML.[29-31] In line with recently published data, our data suggest a prognostic effect of *DNMT3A*$^{\text{mutant}}$ in AML.[14-16] We therefore propose to implement *DNMT3A* mutation analysis in the routine of the molecular diagnostics of human AML.

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Authorship

Contribution: A.F.T.R. and M.P. performed experiments, analyzed data, and wrote the paper; C.E.-V., S.A., and A.Z. performed experiments; V.R. and M.S. analyzed data; M.E.F., A.M., and B.L. analyzed data and wrote the paper; and P.J.M.V. and R.D. designed research, analyzed data, and wrote the paper.

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References

1. Figueroa ME, Lugthart S, Li Y, et al. DNA methylation signatures identify biologically distinct subtypes in acute myeloid leukemia. *Cancer Cell.* 2010;17(1):13-27.
2. Dohner H, Estey EH, Amadori S, et al. Diagnosis and management of acute myeloid leukemia in adults: recommendations from an international expert panel, on behalf of the European LeukemiaNet. *Blood.* 2010;115(3):453-474.
3. Lowenberg B, Downing JR, Burnett A. Acute myeloid leukemia. *N Engl J Med.* 1999;341(14):1051-1062.
4. Li E. Chromatin modification and epigenetic reprogramming in mammalian development. *Nat Rev Genet.* 2002;3(9):662-673.
5. Jaenisch R, Bird A. Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. *Nat Genet.* 2003; 33(Suppl):245-254.
6. Rountree MR, Bachman KE, Herman JG, Baylin SB. DNA methylation, chromatin inheritance, and cancer. *Oncogene.* 2001;20(24):3156-3165.
7. Bestor TH. The DNA methyltransferases of mammals. *Hum Mol Genet.* 2000;9(16):2395-2402.
8. Gowher H, Jeltsch A. Enzymatic properties of recombinant Dnmt3a DNA methyltransferase from mouse: the enzyme modifies DNA in a non-progressive manner and also methylates non-CpG [correction of non-CpA] sites. *J Mol Biol.* 2001;309(5):1201-1208.
9. Toth M, Muller U, Doerfler W. Establishment of de novo DNA methylation patterns. Transcription factor binding and deoxycytidine methylation at CpG and non-CpG sequences in an integrated adenovirus promoter. *J Mol Biol.* 1990;214(3): 673-683.
10. Hsieh CL. Evidence that protein binding specifies sites of DNA demethylation. *Mol Cell Biol.* 1999; 19(1):46-56.
11. Okano M, Bell DW, Haber DA, Li E. DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. *Cell.* 1999;99(3):247-257.
12. Chen ZX, Riggs AD. Maintenance and regulation of DNA methylation patterns in mammals. *Biochem Cell Biol.* 2005;83(4):438-448.
13. Yamashita Y, Yuan J, Suetake I, et al. Array-based genomic resequencing of human leukemia. *Oncogene.* 2010;29(25):3723-3731.
14. Ley TJ, Ding L, Walter MJ, et al. DNMT3A mutations in acute myeloid leukemia. *N Engl J Med.* 2010;16(25):2424-2433.
15. Thol F, Damm F, Ludeking A, et al. Incidence and prognostic influence of DNMT3A mutations in acute myeloid leukemia. *J Clin Oncol.* 2011; 29(21):2889-2896.
16. Yan XJ, Xu J, Gu ZH, et al. Exome sequencing identifies somatic mutations of DNA methyltransferase gene DNMT3A in acute monocytic leukemia. *Nat Genet.* 2011;43(4):309-315.
17. Valk PJ, Bowen DT, Frew ME, Goodeve AC, Lowenberg B, Reilly JT. Second hit mutations in the RTK/RAS signaling pathway in acute myeloid leukemia with inv(16). *Haematologica.* 2004; 89(1):106.
18. Barjesteh van Waalwijk van Doorn-Khosrovani S, Erpelinck C, Meijer J, et al. Biallelic mutations in the CEBPA gene and low CEBPA expression levels as prognostic markers in intermediate-risk AML. *Hematol J.* 2003;4(1):31-40.
19. Care RS, Valk PJ, Goodeve AC, et al. Incidence and prognosis of c-KIT and FLT3 mutations in core binding factor (CBF) acute myeloid leukaemias. *Br J Haematol.* 2003;121(5):775-777.
20. Verhaak RG, Goudswaard CS, van Putten W, et al. Mutations in nucleophosmin (NPM1) in acute myeloid leukemia (AML): association with other gene abnormalities and previously established gene expression signatures and their favorable prognostic significance. *Blood.* 2005; 106(12):3747-3754.
21. Abbas S, Lugthart S, Kavelaars F, et al. Acquired mutations in the genes encoding IDH1 and IDH2 both are recurrent aberrations in acute myeloid leukemia: prevalence and prognostic value. *Blood.* 2010;116(12):2122-2126.
22. Valk PJM, Verhaak RGW, Wouters BJ, et al. Prediction of molecular subtypes in acute myeloid leukemia based on gene expression profiling. *Haematologica.* 2009;94(1):131-134.
23. Taskesen E, Bullinger L, Corbacioglu A, et al. Prognostic impact, concurrent genetic mutations, and gene expression features of AML with CEBPA mutations in a cohort of 1182 cytogenetically normal AML patients: further evidence for CEBPA double mutant AML as a distinctive disease entity. *Blood.* 2011;117(8):2469-2475.
24. Breems DA, Van Putten WL, De Greef GE, et al. Monosomal karyotype in acute myeloid leukemia: a better indicator of poor prognosis than a complex karyotype. *J Clin Oncol.* 2008;26(29):4791-4797.
25. Figueroa ME, Abdel-Wahab O, Lu C, et al. Leukemic IDH1 and IDH2 mutations result in a hypermethylation phenotype, disrupt TET2 function, and impair hematopoietic differentiation. *Cancer Cell.* 2010;18(6):553-567.
26. Alcalay M, Tiacci E, Bergomas R, et al. Acute myeloid leukemia bearing cytoplasmic nucleophosmin (NPMc+ AML) shows a distinct gene expression profile characterized by up-regulation of genes involved in stem-cell maintenance. *Blood.* 2005;106(3):899-902.
27. Becker H, Marcucci G, Maharry K, et al. Favorable prognostic impact of NPM1 mutations in older patients with cytogenetically normal de novo acute myeloid leukemia and associated gene- and microRNA-expression signatures: a Cancer and Leukemia Group B study. *J Clin Oncol.* 2010;28(4):596-604.
28. Vassiliou GS, Cooper JL, Rad R, et al. Mutant nucleophosmin and cooperating pathways drive leukemia initiation and progression in mice. *Nat Genet.* 2011;43(5):470-475.
29. Burnett A, Wetzler M, Lowenberg B. Therapeutic advances in acute myeloid leukemia. *J Clin Oncol.* 2011;29(5):487-494.
30. Marcucci G, Haferlach T, Dohner H. Molecular genetics of adult acute myeloid leukemia: prognostic and therapeutic implications. *J Clin Oncol.* 2011;29(5):475-486.
31. Grimwade D, Walker H, Oliver F, et al. The importance of diagnostic cytogenetics on outcome in AML: analysis of 1,612 patients entered into the MRC AML 10 trial. The Medical Research Council Adult and Children's Leukaemia Working Parties. *Blood.* 1998;92(7):2322-2333.