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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

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We evaluated concurrent gene mutations, clinical outcome, and gene expression signatures of CCAAT/enhancer binding protein alpha (*CEBPA*) double mutations (*CEBPA*^{dm}) versus single mutations (*CEBPA*^{sm}) in 1182 cytogenetically normal acute myeloid leukemia (AML) patients (16-60 years of age). We identified 151 (12.8%) patients with *CEBPA* mutations (91 *CEBPA*^{dm} and 60 *CEBPA*^{sm}). The incidence of germline mutations was 7% (5 of 71), including 3 C-terminal mutations. *CEBPA*^{dm} patients had a lower frequency of concurrent mutations than *CEBPA*^{sm} patients ($P < .0001$). Both, groups were associated with a favorable outcome compared with *CEBPA*^{wt} (5-year overall survival [OS] 63% and 56% vs 39%; $P < .0001$ and $P = .05$, respectively). However, in multivariable analysis only *CEBPA*^{dm} was a prognostic factor for favorable OS outcome (hazard ratio [HR] 0.36, $P < .0001$; event-free survival, HR 0.41, $P < .0001$; relapse-free survival, HR 0.55, $P = .001$). Outcome in *CEBPA*^{sm} is dominated by concurrent *NPM1* and/or *FLT3* internal tandem duplication mutations. Unsupervised and supervised GEP analyses showed that *CEBPA*^{dm} AML (n = 42), but not *CEBPA*^{sm} AML (n = 18), expressed a unique gene signature. A 25-probe set prediction signature for *CEBPA*^{dm} AML showed 100% sensitivity and specificity. Based on these findings, we propose that *CEBPA*^{dm} should be clearly defined from *CEBPA*^{sm} AML and considered as a separate entity in the classification of AML. (*Blood*. 2011;117(8):2469-2475)

Introduction

In the current World Health Organization classification of acute myeloid leukemia (AML), AML with mutated CCAAT/enhancer binding protein alpha (AML with mutated *CEBPA*), has been designated as a provisional disease entity in the category "AML with recurrent genetic abnormalities."[1,2]

CEBPA encodes a transcription factor that is essential for neutrophil development. Targeted disruption of *Cebpa* in mice results in a selective block in early granulocyte development, which is a hallmark of AML.[3,4] Two proteins may be translated from the *CEBPA* transcripts, such as a 42-kDa (p42) and a shorter 30-kDa (p30) protein both translated from the same mRNA transcript. The p42 isoform contains 2 regulatory transactivation domains (TAD) in the N-terminus (TAD1 and TAD2), whereas the shorter p30 isoform only carries the TAD2 domain. Both isoforms contain the C-terminal basic DNA-binding domain and the leucine zipper (bZIP), involved in DNA binding and protein dimerization. In AML, *CEBPA* mutations mainly occur in cytogenetically normal AML (CN-AML) with an incidence of 5% to 14%.[5-14] The 2 main types of mutations can be distinguished: N-terminal frame-shift mutations resulting in the translation of a 30-kDa protein only and the C-terminal in-frame mutations in the basic zipper region affecting DNA binding and homodimerization and heterodimerization.[8,15] As a consequence, these mutations create an imbalance between proliferation and differentiation of hematopoietic progenitors.[10,16]

Patients who have AML with *CEBPA* mutations can be separated into 2 subgroups, namely, those with a single mutation *CEBPA* (*CEBPA*^{sm}) and those with a double mutation *CEBPA* (*CEBPA*^{dm}).[17-21] In the majority of *CEBPA*^{dm} AML, both alleles are mutated.[19] These biallelic mutations frequently consist of an N-terminal mutation on one allele and a C-terminal bZIP mutation on the other. In *CEBPA*^{sm} AML, mutations occur either in the N terminus or in the C terminus of the gene. In previous studies in which these 2 subgroups were not considered, AML patients with mutated *CEBPA* had a relatively good outcome.[5,7,12,13] More recent data suggest that this favorable outcome is mainly observed in AML patients with *CEBPA*^{dm} and not *CEBPA*^{sm}.[17-21] Moreover, it has been suggested that concurrent mutations may occur more frequently in *CEBPA*^{sm} than in *CEBPA*^{dm} AML. The impact of coexisting mutations remains elusive and needs to be validated in large cohorts.

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Table 1. Patient demographics and clinical and molecular characteristics of *CEBPA*^{wt}, *CEBPA*^{sm}, and *CEBPA*^{dm} CN-AML cases

Characteristic	*CEBPA*^{wt} (n = 1031)	*CEBPA*^{sm} (n = 60)	*P*, *CEBPA*^{sm} vs *CEBPA*^{wt}	*CEBPA*^{dm} (n = 91)	*P*, *CEBPA*^{dm} vs *CEBPA*^{wt}	*P*, *CEBPA*^{sm} vs *CEBPA*^{dm}
Median age, y (range)	48 (16-60)	46 (18-60)	.28	44 (16-60)	.04*	.66
Sex, n (%)			.79		.74	.74
Male	500 (48)	28 (47)		46 (51)		
Female	531 (52)	32 (53)		45 (49)		
WBC count, ×10^9/L			.23		.062	.86
Median (range)	28 (0.2-372)	25 (1.1-345)		28 (1.5-262)		
Missing	34	1		4		
Platelet count, ×10^9/L			.77		< .0001*	< .0001*
Median (range)	65 (5-746)	62 (10-361)		38 (4-265)		
Missing	40	3		4		
Bone marrow blasts			.83		.53	.76
Median (range)	80 (0-100)	80 (0-97)		78 (2-100)		
Missing	80	7		4		
Molecular abnormalities						
FLT3^{ITD}, n (%)	347 (33.7)	18 (30)	1	7 (7.7)	< .0001*	.00015*
Missing	69	9		5		
FLT3^{TKD}, n (%)	95 (9.2)	4 (6.7)	.81	2 (2.2)	.018*	.2
Missing	48	6		3		
NPM1, n (%)	560 (54.3)	21 (35)	.018*	3 (3.3)	0	< .0001*
Missing	88	10		8		

Number of cases (percentage), median, range, or missing values are indicated.
WBC indicates white blood cell.
*$P < .05$ computed using the Mann-Whitney *U* test (continuous variables) and 2-sided Fisher exact test (categorical variables).

By applying gene expression profiling (GEP), it was demonstrated that *CEBPA*^{dm} AML can be distinguished from *CEBPA*^{sm} and the majority of *CEBPA*^{wt} AML based on a signature.[18] However, a *CEBPA*^{dm} GEP signature did not predict *CEBPA*^{dm} AML with maximum accuracy because AML in which *CEBPA* was silenced (*CEBPA*^{silenced}) by promoter hypermethylation carried a highly similar signature.[22,23]

The objectives of this study were to evaluate the impact of *CEBPA*^{dm} versus *CEBPA*^{sm} on clinical outcome of CN-AML and to investigate the impact of concurrent mutations in nucleophosmin (*NPM1*^{mutant}) and/or fms-like tyrosine kinase receptor-3 (*FLT3*) internal tandem duplication mutations (*FLT3*^{ITD}). In addition, we searched for *CEBPA*-associated gene expression signatures and determined the frequency of predisposing *CEBPA* germline mutations. For these purposes, we combined datasets from the Dutch-Belgian Hemato-Oncology Cooperative Group (HOVON)-Swiss Group for Clinical Cancer Research (SAKK) and the German and Austrian AML Study Group (AMLSG).

Methods

Patients and molecular analyses

Diagnostic bone marrow or peripheral blood samples from 1182 younger adults (16 to 60 years of age) with CN-AML were analyzed; 193 patients were enrolled on HOVON-SAKK protocols -04, -04A, -29, and -42 (available at www.hovon.nl),[24-27] and 989 patients on AMLSG protocols AML HD93 (n = 74),[28] AML HD98A (n = 313),[29] AMLSG 07-04 (n = 376), registered at www.clinicaltrials.gov as NCT00151242, AML SHG 02-95 (n = 94),[30] and AML SHG 01-99 (n = 180), registered at www.clinicaltrials.gov as NCT00209833). All patients provided written informed consent in accordance with the Declaration of Helsinki. All trials were approved by the Institutional Review Board of Erasmus University Medical Center, University of Ulm, and Hannover Medical School.

Mutation analyses for the genes *FLT3*^{ITD} and *FLT3* tyrosine kinase domain (TKD) mutations (*FLT3*^{TKD}) and the *NPM1* were performed as described previously.[31-33] *CEBPA*^{dm} and *CEBPA*^{sm} AML were identified by denaturing high-performance liquid chromatography or direct sequencing as described.[18] Cases that carried an insertion polymorphism[18,21] (www.ncbi.nlm.nih.gov/sites/snp; genome.ucsc.edu/cgi-bin/hgGateway; www.ensembl.org/Homo_sapiens/Gene/Variation_Gene) or variations that did not lead to amino acid changes were considered wild-type (wt). Cases were categorized as *CEBPA*^{dm} when 2 different mutations or 1 homozygous mutation were present as determined by sequencing analysis; cases with only a single heterozygous mutation were designated as *CEBPA*^{sm}. In 71 of the 151 patients with *CEBPA* mutations, DNA obtained from buccal swabs (n = 52), peripheral blood (n = 8), or bone marrow (n = 11) samples in complete remission (CR) were studied for the presence of *CEBPA* germline mutations. Patient demographics and molecular characteristics are summarized in Table 1. All *CEBPA*-mutated patients, except for patients treated within the AMLSG protocol 07-04, have been previously reported in different studies.[7,13,18]

Gene expression profiling

Data from GEP analysis were available in 674 AML patients (53% CN-AML, HOVON-SAKK and AMLSG cohorts), generated using Affymetrix (supplemental Table 1, available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). Sample processing and quality control were carried out as described previously.[23,34] For both cohorts, normalization of raw data was processed with Affymetrix Microarray Suite 5 (MAS5) to target intensity values at 100. Intensity values were $\log_2$ transformed, and mean centered per probe set per cohort. GEP data are available at the Gene Expression Omnibus (National Center for Biotechnology Information; accession number GSE14468 [HOVON-SAKK cohort] and GSE22845 [AMLSG cohort]). There were 42 *CEBPA*^{dm} and 18 *CEBPA*^{sm} cases for which the GEP was determined (supplemental Table 1).

Statistical analyses

Statistical analyses were performed using Mathworks (Matlab R2009b) with the statistical, bioinformatics, and pattern recognition toolbox (Prtools). For clinical, molecular, univariate, and multivariate analyses, patients with CN-AML and age ≤ 60 years (supplemental Table 1) were included. Molecular and clinical variables of both patient cohorts (HOVON-SAKK and AMLSG) were comparable. Differences were assessed for *CEBPA*^{sm} and *CEBPA*^{dm} groups in comparison with *CEBPA*^{wt} group (Table 1), using the Mann-Whitney *U* test for continuous variables and the 2-sided Fisher exact test for categorical variables.

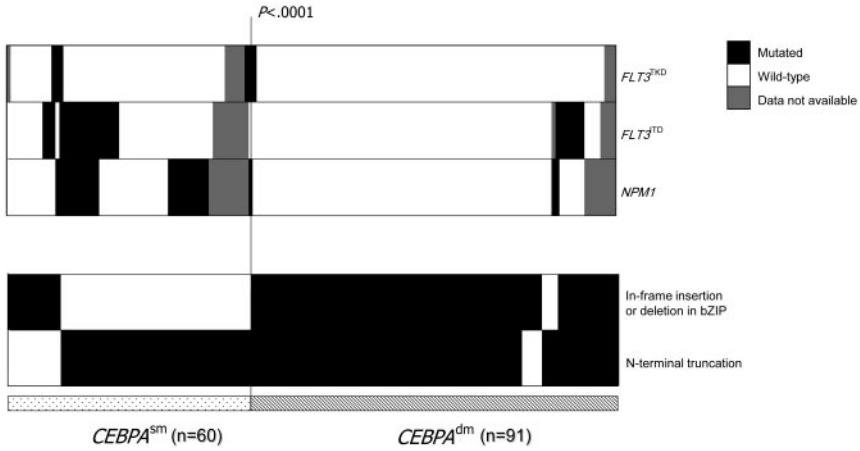

Figure 1. Distribution of concurrent mutations in *CEBPA*^{dm} and *CEBPA*^{sm} patients. Columns represent patients with *CEBPA* mutations (91 *CEBPA*^{dm} and 60 *CEBPA*^{sm}) patients and rows represent the genotypes *FLT3*^{TKD}, *FLT3*^{ITD}, and *NPM1*^{mutant} (filled black), wild-type (open), or missing (filled gray). The in-frame insertion or deletion in bZIP and N-terminal truncation mutations in *CEBPA* is shown and highlighted in black.

Outcome measures of the HOVON-SAKK and AMLSG cohorts were comparable (log-rank test overall survival [OS], $P = .08$; event-free survival [EFS], $P = .47$; supplemental Figure 1A-B, for respective cohort). There were no statistical differences in outcome in patients receiving autologous or allogeneic hematopoietic stem cell transplantation between the HOVON-SAKK and AMLSG cohorts (log-rank test OS, $P = .68$; EFS, $P = .89$; supplemental Figure 2A-B, respectively).

For univariate analysis, significance was assessed using the stratified log-rank test and Kaplan-Meier estimates for OS, EFS and relapse-free survival (RFS). The recommended consensus criteria[35] were used for the definition of CR and survival end points such as OS, EFS, and RFS. Multivariate analysis was performed using stratified Cox proportional hazard model. For all analyses, a value for $P \leq .05$ was considered statistically significant and for survival analyses, values for P were computed using the full time span. Note that the closed testing procedure[36] was applied, and a correction for multiple testing[37] was only performed if the global log-rank test resulted in $P > .05$.

For gene expression–based classification of *CEBPA*^{dm} cases, GEP of the HOVON-SAKK cohort was used to derive the 25-probe set predictive signature and the AMLSG cohort as validation set. To summarize, a logistic regression model with Lasso regularization (a continuous feature selection procedure) was used because it takes the correlation structure between the probe sets into account (see supplemental "creation and evaluation of the *CEBPA*^{dm} predictive signature").

Results

Frequency and type of acquired *CEBPA*^{dm} and *CEBPA*^{sm} mutations

CEBPA mutations were detected in 151 of the 1182 (12.8%) CN-AML patients; 91 (60%) had *CEBPA*^{dm} among which the combination of N- and C-terminal mutations was the predominant genotype (82 of the 91). *CEBPA*^{dm} cases with only N- or C-terminal mutations were less frequently observed (4 of the 91 or 5 of the 91, respectively). A total of 60 of 151 (40%) *CEBPA*-mutated cases had *CEBPA*^{sm}, which occurred most frequently in the N terminus (47 of the 60). Only 13 of the 60 *CEBPA*^{sm} cases had in-frame insertion or deletion mutations affecting the bZIP domain (Figure 1).

CEBPA germline mutation analysis

Five of 71 (7%) *CEBPA* mutant AML patients analyzed carried *CEBPA* germline mutations: in 2 of the 5 patients, the germline mutation was localized in the N-terminus, and both acquired a C-terminal mutation. Both patients had a family history of AML and were diagnosed at a young age. In the remaining 3 patients, the germline mutation was in the C terminus; 1 of these patients gained an additional N-terminal mutation and the second patient an additional C-terminal mutation at the time of AML-diagnosis. None of these 3 patients had a family history of AML. Alignment to distinct Single Nucleotide Polymorphism databases (www.ncbi.nlm.nih.gov/sites/snp; genome.ucsc.edu; or www.ensembl.org/Homo_sapiens/Gene/Variation_Gene) did not identify one of these germline sequence variations as a polymorphism. Using the PolyPhen database (http://genetics.bwh.harvard.edu/pph/), all 3 C-terminal mutations were predicted to be damaging to the function and structure of the protein (Table 2).

Association of acquired *CEBPA*^{dm} and *CEBPA*^{sm} mutations with concurrent gene mutations and clinical characteristics

Concurrent mutations were seen less frequently in *CEBPA*^{dm} than in *CEBPA*^{sm} AML (22% vs 60%; $P < .0001$, Figure 1); frequency for *NPM1*^{mutant} in *CEBPA*^{dm} versus *CEBPA*^{sm} AML was 3.3% and 35% ($P < .0001$), and for *FLT3*^{ITD} was 7.7% and 30% ($P < .001$), respectively (Table 1). Comparing *CEBPA*^{sm} and *CEBPA*^{wt} AML, *NPM1*^{mutant} were slightly less frequent in *CEBPA*^{sm} AML (35% vs

Table 2. Germline patient demographics and molecular characteristics

Patient ID	Age at diagnosis, y	Germline mutation	Acquired mutation*	Additional mutation†	Familial AML history	*CEBPA* mutation
98A-751	28	338delC	1080insGAA	None	Yes	*CEBPA*^{dm}
07/04-268 (ULM_10)	25	307delG	1122_1123ins1075_1225	*KRAS*, *WT1*	Yes	*CEBPA*^{dm}
BioID 769	51	1096T>C	478_485del	None	No	*CEBPA*^{dm}
98A-543	33	1164G>A	None	*FLT3*^{TKD}, *NPM1*	No	*CEBPA*^{sm}
07/04-48 (ULM_20)	59	1036G>T	1086insAAG	None	No	*CEBPA*^{dm}

Characteristics of 5 of 71 (7%) *CEBPA* mutant AML patients who carried *CEBPA* germline mutations. CBL indicates Casitas B-lineage lymphoma; KRAS, Kirsten Rat sarcoma; NRAS, neuroblastoma Rat sarcoma; RUNX1, runt-related transcription factor 1; and WT1, Wilms tumor 1

*Data according to GenBank accession no. Y11525.

†Patients 98A-751, 07/04-268 (ULM_10), 98A-543, and 07/04-48 (ULM_20) were screened for *FLT3*^{ITD}, *FLT3*^{TKD}, *NPM1*, *NRAS*, *KRAS*, *WT1*, *RUNX1*, and *CBL* mutations; patient BioID 769 was analyzed for *FLT3*^{ITD}, *FLT3*^{TKD}, and *NPM1* mutations.

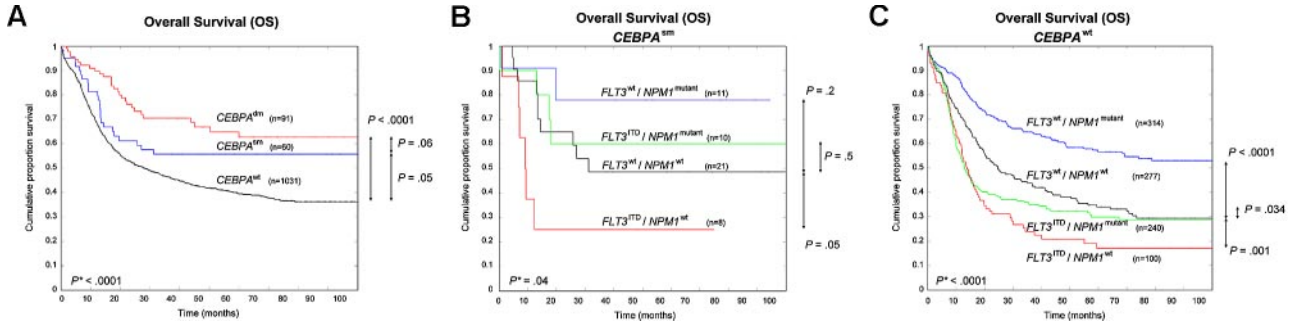

Figure 2. Kaplan-Meier survival curves for OS. (A) Kaplan-Meier survival curves for OS among the 3 groups *CEBPA*^{dm}, *CEBPA*^{sm}, and *CEBPA*^{wt}. (B) Kaplan-Meier survival curves for OS of the 4 genotypes *FLT3*^{ITD}/*NPM1*^{mutant}, *FLT3*^{ITD}/*NPM1*^{wt}, *FLT3*^{wt}/*NPM1*^{mutant}, and *FLT3*^{wt}/*NPM1*^{wt} within the *CEBPA*^{sm} group. (C) Kaplan-Meier survival curves for OS of the 4 genotypes within *CEBPA*^{wt}. **P* value by global log-rank test.

54.3%; $P = .018$); the frequency for *FLT3*^{ITD} was comparable between the 2 groups (30% vs 33.7%).

Regarding presenting clinical characteristics, *CEBPA*^{dm} mutations were associated with younger age (median age 44 vs 48 years; $P = .04$) and lower platelet counts (median number 38×10^9/L vs 65×10^9/L; $P < .0001$) compared with *CEBPA*^{wt} patients (Table 1).

Impact of *CEBPA*^{dm} and *CEBPA*^{sm} on response to induction therapy and clinical outcome

For correlation with clinical outcome, 1182 CN-AML were considered. *CEBPA*^{dm} was associated with a higher CR rate compared with *CEBPA*^{sm} (92% vs 78%, $P = .02$) and with *CEBPA*^{wt} (92% vs 79%, $P = .002$). There was no difference to CR probability between *CEBPA*^{sm} and *CEBPA*^{wt} patients (78% vs 79%, $P = .86$).

The median follow-up time for survival in the 1182 CN-AML patients was 33 months (95% confidence interval [CI], 25.6-0.4); the estimated 5-year OS and RFS were 42% (95% CI, 39%-45%) and 34% (95% CI, 31%-38%), respectively.

CEBPA^{dm} AML was associated with a significantly superior outcome compared with *CEBPA*^{wt} AML (5-year OS, 63% vs 39%, $P < .0001$; EFS, 45% vs 28%, $P < .0001$; RFS, 44% vs 32%, $P = .05$), as shown (Figure 2A and supplemental Figure 3A,D). A somewhat better outcome was also found for *CEBPA*^{sm} AML compared with *CEBPA*^{wt} AML (5-year OS, 55% vs 39%, $P = .05$; RFS, 49% vs 32%, $P = .02$; but not EFS, 37% vs 28%, $P = .22$). No significant difference was evident between *CEBPA*^{dm} and *CEBPA*^{sm} AML (5-year OS, $P = .06$; EFS, $P = .16$; RFS, $P = .48$). Of note, no differences in outcome were observed among *CEBPA*^{sm} patients with either C-terminal (n = 13) or N-terminal (n = 47) mutations (5-year OS, 54% vs 56%, $P = .58$; supplemental Figure 4).

In multivariate analyses considering other prognostic indicators (Table 3), the presence of *CEBPA*^{dm} was an independent prognostic factor for favorable OS (HR 0.36, $P < .0001$), EFS (HR 0.41, $P < .0001$), and RFS (HR 0.55, $P = .001$), whereas *CEBPA*^{sm} did not impact these 3 end points (Table 3).

Treatment outcome of AML with *CEBPA*^{sm} is dominated by *FLT3*/*NPM1* genotypes

Finally, we performed explorative subgroup analyses in *CEBPA*^{sm} and *CEBPA*^{wt} AML to evaluate the impact of 4 *FLT3*/*NPM1* genotype subgroups: *FLT3*^{ITD}/*NPM1*^{mutant} (n = 10); *FLT3*^{ITD}/*NPM1*^{wt} (n = 8); *FLT3*^{wt}/*NPM1*^{mutant} (n = 11); and *FLT3*^{wt}/*NPM1*^{wt} (n = 21). There were 10 cases from the *CEBPA*^{sm} group excluded for which the genotypes were unknown.

Among patients with *CEBPA*^{sm} AML, the *FLT3*^{ITD}/*NPM1*^{wt} genotype had an inferior OS compared with patients with the *FLT3*^{wt}/*NPM1*^{wt} genotype (5-year OS, 25% vs 49%, $P = .05$; Figure 2B); for EFS and RFS, there was a trend toward an inferior outcome (supplemental Figure 3B,E); in contrast, the *FLT3*^{wt}/*NPM1*^{mutant} genotype associated in trend with a favorable outcome compared with the *FLT3*^{wt}/*NPM1*^{wt} genotype (5-year OS, 78% vs 49%, $P = .2$; EFS, 59% vs 32%, $P = .08$; RFS, 66% vs 40%, $P = .38$; Figure 2B and supplemental Figure 3B,E). In analogy, in the *CEBPA*^{wt} group, the *FLT3*^{ITD}/*NPM1*^{wt} genotype had a significantly inferior survival compared with the *FLT3*^{wt}/*NPM1*^{wt} genotype (5-year OS, 17% vs 34%, $P = .001$; EFS, 11% vs 14%, $P = .04$; RFS, 15% vs 24%, $P = .002$; Figure 2C and supplemental

Table 3. Multivariate analysis for overall, event-free, and relapse-free survival in CN-AML patients

Variable*	HR	95% CI	*P*
Overall survival			
CEBPA$^{sm\alpha}$	0.70	0.46-1.07	.10
CEBPA$^{dm\alpha}$	0.36	0.23-0.55	< .0001†
FLT3$^{ITD\beta}$	1.78	1.49-2.14	< .0001†
FLT3$^{TKD\beta}$	0.84	0.61-1.15	.28
NPM1$^{\beta}$	0.56	0.46-0.67	< .0001†
WBC count$^{\delta}$, $\times 10^9$/L	1.35	1.12-1.62	< .0001†
Age$^{\epsilon}$	1.02	1.01-1.03	< .0001†
Event-free survival			
CEBPA$^{sm\alpha}$	0.86	0.60-1.22	.40
CEBPA$^{dm\alpha}$	0.41	0.29-0.57	< .0001†
FLT3$^{ITD\beta}$	1.56	1.33-1.84	< .0001†
FLT3$^{TKD\beta}$	0.80	0.60-1.07	.13
NPM1$^{\beta}$	0.45	0.39-0.53	< .0001†
WBC count$^{\delta}$, $\times 10^9$/L	1.27	1.08-1.50	.003†
Age$^{\epsilon}$	1.01	1.01-1.02	.003†
Relapse-free survival			
CEBPA$^{sm\alpha}$	0.79	0.51-1.22	.30
CEBPA$^{dm\alpha}$	0.55	0.38-0.79	.001†
FLT3$^{ITD\beta}$	1.75	1.45-2.12	< .0001†
FLT3$^{TKD\beta}$	0.82	0.59-1.13	.22
NPM1$^{\beta}$	0.56	0.46-0.68	< .0001†
WBC count$^{\delta}$, $\times 10^9$/L	1.33	1.10-1.61	.002†
Age$^{\epsilon}$	1.01	1.00-1.02	.001†

Stratified Cox proportional hazard ratio (HR) model for multivariable analyses of *CEBPA*^{dm} and *CEBPA*^{sm} as prognostic marker for overall survival, event-free survival, and relapse-free survival. Analyses included 1182 CN-AML patients ≤ 60 years of age.

WBC indicates white blood cell.

*Subgroup α *CEBPA* status vs *CEBPA*^{wt} β *FLT3*^{ITD} vs no *FLT3*^{ITD} mutation β *FLT3*^{TKD} vs no *FLT3*^{TKD} mutation β *NPM1* vs no *NPM1* δ. WBC count > 20×10^9/L vs < 20×10^9/L ϵ. Age is used as a continuous variable.

†$P \leq .05$.

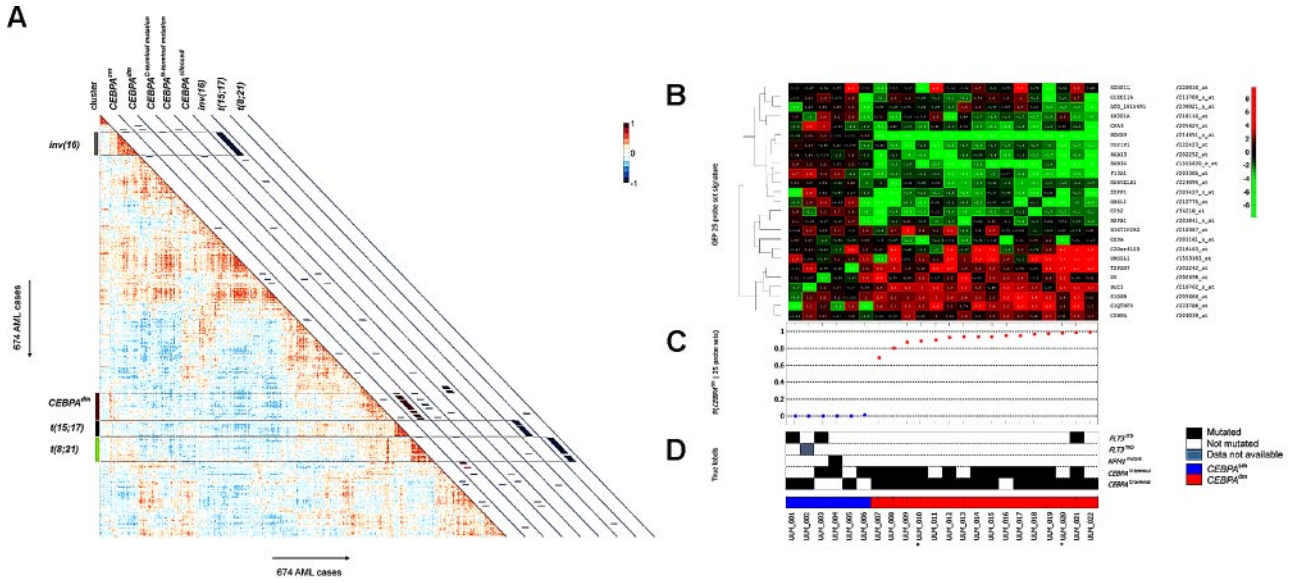

Figure 3. Unsupervised analyses and classification results of candidate *CEBPA*^{dm} cases with their GEP and their molecular characteristics. (A) Pairwise correlations between the 674 AML cases (supplemental Table 1). The cells in the visualization are colored by Pearson correlations values, depicting higher positive (red) or negative (blue) correlations, as indicated by the scale bar. *CEBPA*^{sm}, *CEBPA*^{dm}, *CEBPA*$^{C\text{-terminal mutation}}$, *CEBPA*$^{N\text{-terminal mutation}}$, *CEBPA*^{silenced}, together with inv(16), t(15;17), and t(8;21) cases are depicted on the diagonal with a red or blue colored bar. *CEBPA*$^{C\text{-terminal mutation}}$ and *CEBPA*$^{N\text{-terminal mutation}}$ indicates the presence of homozygous mutations. (B) Candidate *CEBPA*^{dm} patients and the unambiguous *CEBPA*^{sm} patients. The expression levels are defined by the 25-probe set signature. The colors of the hierarchical clustering are relative to the mean. (C) Computed posterior probabilities, indicating the prediction of a *CEBPA*^{dm} case, given the 25 predictive probe set signature: *P*(*CEBPA*^{dm} 25-probe sets). The ordering of patients is based on the classification probabilities. (D) True labels (molecular characteristics). Filled lanes indicate the mutation status in *CEBPA* (*CEBPA*^{dm} or *CEBPA*^{sm}), *NPM1* (*NPM1*^{mutant}), or *FLT3* (TKD or ITD), open lanes represents no mutation in the particular patient, and a missing value is depicted in gray. *Germline *CEBPA*^{dm} cases.

Figure 3C,F), whereas the *FLT3*^{wt}/*NPM1*^{mutant} genotype was associated with a favorable outcome (5-year OS, 57% vs 34%, $P < .0001$; EFS, 47% vs 14%, $P < .0001$; RFS, 50% vs 24%, $P < .0001$; Figure 2C and supplemental Figure 3C,F). Thus, we observed comparable trends for favorable (*FLT3*^{wt}/*NPM1*^{mutant}) and inferior (*FLT3*^{ITD}/*NPM1*^{wt}) outcome in the *CEBPA*^{sm} and *CEBPA*^{wt} subgroups. The outcome for all *CEBPA*^{sm} *FLT3*/*NPM1* genotypes was higher (not significantly, $P > .05$), compared with the *CEBPA*^{wt} genotypes; however, the distinct groups were relatively small. For *CEBPA*^{dm} AML, sample sizes of the composite genotypic subgroups were too small for analysis.

Unsupervised analyses of GEP showed homogeneity in *CEBPA*^{dm} AML cases

GEP was performed in a subset of the CN-AML patients and includes cytogenetically abnormal patients (n = 674) as shown (supplemental Table 1). Unsupervised analyses, by computing pairwise Pearson correlation coefficients of 674 AML cases, revealed distinct GEP clusters (Figure 3A), including the known clusters of AML with inv(16), t(15;17), or t(8;21), as shown previously.[23] These subtypes revealed high correlation within the GEP cluster (average correlation, 0.42, 0.49, and 0.49, respectively) and differed significantly ($P < .0001$) among the AML cases without any of these aberrations, (supplemental Figure 5B-C and supplemental Figure 5E). We observed that the *CEBPA*^{dm} AML cases were highly similar within the cluster (average correlation, 0.35) and differed significantly from cases without a *CEBPA*^{dm} ($P < .0001$; supplemental Figure 5D). *CEBPA*^{sm} AML cases showed reduced similarity (average correlation, 0.15) and did not differ from cases without *CEBPA*^{sm} ($P = .12$; supplemental Figures 3A,5A).

CEBPA^{dm} AML is accurately predicted based on GEP

The previously predictive *CEBPA*^{dm} signature[18] was hampered by the recently reported *CEBPA*^{silenced} AML cases that carry a similar GEP.[22] The 2 independent AML cohorts were used to train and evaluate the predictive value of the *CEBPA*^{dm} signature in terms of sensitivity and specificity. A predictive signature was created (Figure 3B and supplemental Table 2), containing 25-probe sets, using a logistic regression model with Lasso regularization[38,39] that selects discriminative probe sets between the classes, *CEBPA*^{dm} (n = 26) and all other AML cases, *CEBPA*^{wt} and *CEBPA*^{sm} (n = 494). Subsequently, a classifier was trained on the entire HOVON-SAKK cohort based on a 2-class approach; with 26 *CEBPA*^{dm} versus 494 other (*CEBPA*^{wt} and *CEBPA*^{sm}) cases. This trained classifier subsequently classified 16 candidate *CEBPA*^{dm} cases (supplemental Table 3) in the AMLSG cohort of 154 AML cases (16 *CEBPA*^{dm}, 6 *CEBPA*^{sm}, and 132 *CEBPA*^{wt}; supplemental Table 1). Among the *CEBPA*^{dm} cases were 5 with either homozygous N- or C-terminal *CEBPA*^{dm} mutations, and a *CEBPA*^{dm} patient with a germline C-terminal mutation. This approach showed perfect sensitivity and specificity (both 100%; Figure 3C). In addition, we performed a classification among *CEBPA*^{dm}, *CEBPA*^{sm}, and *CEBPA*^{wt} to infer whether we were able to accurately classify *CEBPA*^{sm} cases. We observed that all *CEBPA*^{sm} cases were classified as *CEBPA*^{wt}, thus *CEBPA*^{sm} cases did not have a consistent gene expression pattern and were different from the *CEBPA*^{dm} group.

Discussion

In this study, we established the value of *CEBPA*^{dm} mutation in a large cohort of CN-AML patients from AMLSG and HOVON-SAKK treatment trials. Applying denaturing high-performance liquid chromatography and whole gene sequencing, we detected 91 (7.7%) *CEBPA*^{dm} and 60 (5.1%) *CEBPA*^{sm} mutations among 1182 patients. In multivariate analyses, we demonstrate that the presence of *CEBPA*^{dm} but not *CEBPA*^{sm} is an independent factor for favorable outcome in AML, which confirms previous findings reported in studies with relatively small cohorts.[17-19,21]

Concurrent mutations were significantly less frequent in *CEBPA*^{dm} compared with *CEBPA*^{sm} AML. This factor was true for *FLT3*^{ITD} and in particular for *NPM1*^{mutant}, which were virtually not present among *CEBPA*^{dm} cases, a finding that is consistent with previously published data.[20]

Compared with previous studies[17-21] and with the large number of cases, we were able to evaluate the prognostic impact of the *CEBPA* mutational status in the context of the *FLT3*/*NPM1* genotypes. Among *CEBPA*^{sm} AML, the 4 combined genotypes showed similar trend with regard to outcome compared with *CEBPA*^{wt} AML (Figure 2B-C). Nevertheless, we observed a higher outcome (not significant) for all *CEBPA*^{sm} *FLT3*/*NPM1* genotypes compared with the *CEBPA*^{wt} genotypes, but these groups are relatively small. These findings, supported by data from multivariable analysis, strongly suggest that not the existence of *CEBPA*^{sm} per se but rather the combined effects of *CEBPA*^{sm} and *FLT3*^{ITD} and/or *NPM1*^{mutant} determine outcome in these AML patients.

We have previously derived gene expression signatures that predict AML with inv(16), t(15;17), and t(8;21) with 100% accuracy. In this study, we generated a refined GEP signature of 25-probe sets that predict *CEBPA*^{dm} AML cases (6 genes overlapped with the previous signature,[18] as indicated in "Supplemental Materials"). This signature showed sensitivity and specificity of 100% and has a better predictive power than the *CEBPA*^{dm} signature that we defined before.[18] In fact, in contrast to the previous signature, the new signature also discriminates *CEBPA*^{dm} from AML with hypermethylation of the proximal promoter region of *CEBPA*.[22] Classification results were not affected by homozygous N- or C-terminal *CEBPA*^{dm} mutations or because of germline mutation. Because this 25-probe set signature was optimized for classification it does not necessarily provide insight into the biologic meaning of *CEBPA*^{dm} mutations.

Currently, nucleotide sequencing is used as the gold standard for the identification of *CEBPA* mutations. Because of the much higher effort required, the GEP technique should not be considered as a primary diagnostic tool in AML. However, GEP can be confirmatory, especially in cases in which the *CEBPA* gene appears difficult to sequence. More importantly, GEP provides relevant insights in the biology of the disease and the affected signaling pathways and therefore allows further classification/refinement of AML.

Finally, we evaluated the frequency of *CEBPA* germline mutations in this large cohort of *CEBPA*-mutated cases. Among 71 mutated patients, 5 revealed germline mutations. Of these cases, 4 developed *CEBPA*^{dm} AML, that is, 4 cases acquired a mutation in the second allele. This finding is in line with previous data.[40,41] Interestingly for the first time, we identified 3 C-terminal germline mutations of which 2 cases acquired a second *CEBPA* mutation at the time of AML diagnosis. In GEP analysis both cases clustered within the *CEBPA*^{dm} group and were classified as a *CEBPA*^{dm}, providing evidence that these C-terminal sequence variations are mutations rather than polymorphisms. In line with the GEP data, all 3 C-terminal germline mutations were predicted to be damaging for the function and the structure of the protein.

In the current World Health Organization classification of AML, AML with mutated *CEBPA* has been designated as a provisional disease entity in the category "AML with recurrent genetic abnormalities." Based on our data obtained from a large patient cohort together with previous findings, we propose that *CEBPA*^{dm} AML should be clearly distinguished from *CEBPA*^{sm} AML and that only AML with *CEBPA*^{dm} should be considered as an independent entity in the classification of the disease.

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Authorship

Contribution: E.T. performed research, data analysis, data interpretation, creation of the figures, and manuscript writing; L.B. and A.C. performed research, data analysis, and interpretation; M.A.S. performed data analysis, data interpretation, and manuscript writing; C.A.J.E., B.J.W., and S.C.v.d.P.-v.d.L. performed research; F.D. performed research and data interpretation; J.K. and A.G. provided study material; R.F.S. performed research, data interpretation, and manuscript writing; and B.L., R.D., H.D., P.J.M.V., and K.D. designed the study and performed data interpretation and manuscript writing.

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