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ORIGINAL REPORT

Acute Myeloid Leukemia With Biallelic *CEBPA* Gene Mutations and Normal Karyotype Represents a Distinct Genetic Entity Associated With a Favorable Clinical Outcome

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ABSTRACT

Purpose
CEBPA mutations are found as either biallelic (bi*CEBPA*) or monoallelic (mo*CEBPA*). We set out to explore whether the kind of *CEBPA* mutation is of prognostic relevance in cytogenetically normal (CN) acute myeloid leukemia (AML).

Patients and Methods
Four hundred sixty-seven homogeneously treated patients with CN-AML were subdivided into mo*CEBPA*, bi*CEBPA*, and wild-type (wt) *CEBPA* patients. The subgroups were analyzed for clinical parameters and for additional mutations in the *NPM1*, *FLT3*, and *MLL* genes. Furthermore, we obtained gene expression profiles using oligonucleotide microarrays.

Results
Only patients with bi*CEBPA* had an improved median overall survival when compared with patients with wt*CEBPA* (not reached *v* 20.4 months, respectively; $P = .018$), whereas patients with mo*CEBPA* (20.9 months) and wt*CEBPA* had a similar outcome ($P = .506$). Multivariable analysis confirmed bi*CEBPA*, but not mo*CEBPA*, mutations as an independent favorable prognostic factor. Interestingly, bi*CEBPA* mutations, compared with wt*CEBPA*, were never associated with mutated *NPM1* (0% *v* 43%, respectively; $P < .001$) and rarely associated with *FLT3* internal tandem duplication (ITD; 5% *v* 23%, respectively; $P = .059$), whereas patients with mo*CEBPA* had a similar frequency of mutated *NPM1* and a significantly higher association with *FLT3*-ITD compared with patients with wt*CEBPA* (44% *v* 23%, respectively; $P = .037$). Furthermore, patients with bi*CEBPA* showed a homogeneous gene expression profile that was characterized by downregulation of *HOX* genes, whereas patients with mo*CEBPA* showed greater heterogeneity in their gene expression profiles.

Conclusion
Biallelic disruption of the N and C terminus of *CEBPA* is required for the favorable clinical outcome of *CEBPA*-mutated patients and represents a distinct molecular subtype of CN-AML with a different frequency of associated gene mutations. These findings are of great significance for risk-adapted therapeutic strategies in AML.

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INTRODUCTION

Cytogenetically normal (CN) acute myeloid leukemia (AML) represents a molecularly heterogeneous disease entity in which mutations in certain genes have been linked to clinical outcome. Mutations in the *CEBPA* and *NPM1* genes, internal tandem duplications (ITD) of the fms-like tyrosine kinase 3 (*FLT3*) gene, and partial tandem duplications (PTD) of the *MLL* gene are the most common prognostic markers.[1] Accordingly, the recently revised fourth edition of the *WHO Classification of Tumours of the Haematopoietic and Lymphoid Tissues* strongly recommends a routine mutational screen of *NPM1*, *CEBPA*, and *FLT3*-ITD and includes AML with mutated *NPM1* and AML with mutated *CEBPA* as provisional entities.[2]

The *CEBPA* gene located on chromosome 19 band q13.1 encodes a member of the basic region

leucine zipper (bZIP) transcription factor family, which coordinates myeloid differentiation and cellular growth arrest.[3] We and others have reported *CEBPA* mutations in 8% to 19% of patients with CN-AML.[1,4-7] The mutations are typically associated with CN-AML,[7,8] French-American-British AML subtypes M1 or M2, and a better overall survival (OS).[1,5,6] *CEBPA* mutations have largely been divided into the following two main categories: N-terminal frameshift mutations that specifically abolish the translation of the full-length (42-kDa) CEBPA protein (p42 CEBPA), leading to the overexpression of a shorter, dominant negative 30-kDa isoform of CEBPA (p30 CEBPA).[7] C-terminal in-frame mutations in the bZIP domain of *CEBPA* lead to proteins with disrupted homo- and heterodimerization domains and consequently impaired DNA binding activities.[8,9]

Most *CEBPA*-mutated patients with AML carry two mutations on separate alleles of *CEBPA* with a specific combination of an N-terminal frameshift mutation on one allele and a C-terminal in-frame mutation on the other allele.[10] Furthermore, AML blasts from patients with biallelic *CEBPA* mutations (bi*CEBPA*) have been associated with a distinct immunophenotype.[11] Therefore, we wanted to investigate, in a large and homogeneously treated cohort of patients with CN-AML, whether patients with bi*CEBPA* form a clinically and molecularly distinct group that can be separated from patients with

Table 1. Patient Demographics and Clinical and Molecular Characteristics of CN-AML Patients According to the Number of Mutated Alleles in *CEBPA*

Characteristic	wt*CEBPA* (n = 429)		mo*CEBPA* (n = 18)		bi*CEBPA* (n = 20)		*P**
	No. of Patients	%	No. of Patients	%	No. of Patients	%	
Age, years							.693
Median	61		65		62		
Range	17-85		18-78		28-75		
Female	192†	45	15	83	9†	45	.006
FAB type (n = 447)							.335
M0	16	4	0	0	0	0	
M1	83	20	5	28	6	30	
M2	162	40	9	50	12	60	
M3	0	0	0	0	0	0	
M4	90	22	3	17	1	5	
M5	37	9	0	0	0	0	
M6	21	5	1	6	1	5	
M1/M2 (n = 447)	245	60	14	78	18†	90	.009
Type of disease (n = 466)							.613
De novo AML	332	78	14	78	19	95	
AML from MDS	67	16	3	17	1	5	
Therapy-related AML	14	3	1	6	0	0	
High-risk MDS‡	15	4	0	0	0	0	
Allogeneic stem-cell transplantation	89	21	4	22	5	25	.893
Hemoglobin, g/L (n = 457)							.035
Median	93		94		103†		
Range	47-164		72-131		86-125		
WBC count, $\times 10^9$/L (n = 460)							.158
Median	9.6		19.0		10.5		
Range	0.5-170.0		1.7-128.9		0.9-103.5		
Platelet count, $\times 10^9$/L, n = 460							.303
Median	58		50		40		
Range	3-643		10-223		18-176		
Bone marrow blasts, % (n = 439)							.118
Median	75		85		80		
Range	10-100		20-97		30-95		
LDH, U/L (n = 458)							.378
Median	372		398		284		
Range	8-14,332		152-2,660		205-1,238		
Mutated *NPM1* (n = 413)	162/375§	43	8	41	0†	0	.001
FLT3-ITD (n = 447)	94/409†	23	8	44	1†	5	.014
FLT3-TKD (n = 379)	24/346	7	1/15	7	0/18	0	.720
MLL-PTD (n = 426)	34/391	9	1/17	6	0/18	0	.611

Abbreviations: CN-AML, cytogenetically normal acute myeloid leukemia; wt*CEBPA*, wild-type *CEBPA*; mo*CEBPA*, monoallelic *CEBPA* mutation; bi*CEBPA*, biallelic *CEBPA* mutations; FAB, French-American-British; AML, acute myeloid leukemia; MDS, myelodysplastic syndrome; LDH, lactate dehydrogenase; *NPM1*, nucleophosmin; *FLT3*-ITD, internal tandem duplication of the *FLT3* gene; *FLT3*-TKD, tyrosine kinase domain mutations; *MLL*-PTD, partial tandem duplication of the *MLL* gene.

*Differences between wt*CEBPA*, mo*CEBPA*, and bi*CEBPA* were calculated using the Kruskal-Wallis test or χ^2 test/Fisher's exact test.

†Statistically significant also in pairwise comparison to mo*CEBPA* applying the Mann-Whitney *U* test or χ^2 test/Fisher's exact test.

‡Blasts = 10% to 20%.

§No./total No. with available data.

monoallelic *CEBPA* mutations (mo*CEBPA*). We show here that biallelic, but not monoallelic, disruption of *CEBPA* is required for a favorable outcome and that these patients represent a distinct molecular subgroup within AML with mutated *CEBPA* with a characteristic gene expression profile.

PATIENTS AND METHODS

Patients

In this analysis, we included diagnostic bone marrow or peripheral-blood samples from 467 adults with a normal karyotype and with a median age of 61 years who were enrolled onto the German Acute Myeloid Leukemia Cooperative Group (AML CG) 1999 multicenter treatment trial.[12] Details regarding patients, patient selection, and treatment regimens are described in the Appendix and in Appendix Table A1 (online only). The study protocols were approved by the ethics committees of the participating centers, and all patients provided written informed consent.

Molecular Analyses

CEBPA mutational screening was performed using a multiplex polymerase chain reaction–based fragment length analysis as described.[4] For all samples harboring more than one *CEBPA* mutation, the full-length *CEBPA* open reading frames were cloned as described[4] to clarify whether the different mutations were on the same or on separate alleles. Most samples were further characterized at the molecular level with regard to *NPM1* mutations,[13] *FLT3*-ITD,[14] activation-loop mutations at D835 in the *FLT3* gene (*FLT3*-TKD),[15] and *MLL*-PTD,[16] as previously described.

Gene Expression Profiling

We used gene expression profiles from 61 patients with known *CEBPA* status, obtained using Affymetrix HG-U133A microarrays (Affymetrix, Santa Clara, CA), to analyze differences in gene expression between patients with bi*CEBPA* and those with mo*CEBPA* or wild-type (wt) *CEBPA*. Detailed methods for microarray analysis are available in the Appendix (online only).

Statistical Analyses

Clinical and molecular baseline characteristics were compared between the three *CEBPA* groups using the Kruskal-Wallis test (continuous variables) and the χ^2 test (categoric variables). Additional pairwise comparisons were performed after the closed testing procedure only for significant three-group comparisons,[17] using the Mann-Whitney *U* test for continuous variables and the χ^2 test for categoric variables. Kaplan-Meier estimates were calculated for time-to-event variables. Follow-up time was estimated using the reverse Kaplan-Meier method.[18] Outcome of mo*CEBPA* and bi*CEBPA* patients was compared pairwise to outcome of wt*CEBPA* patients using the Mann-Whitney *U* test, χ^2 test, or log-rank test (time-to-event variables). Multivariable analysis was performed using the Cox proportional hazards model. Further details are given in the Appendix.

RESULTS

Molecular Characterization of Patients With moCEBPA and biCEBPA Mutations

We recently reported a *CEBPA* mutation screen of 467 samples from patients with CN-AML. In this cohort, 58 *CEBPA* mutations in 38 patients (8%) were identified.[4] Approximately half of the patients (20 of 38 patients; 52%) had two *CEBPA* mutations, whereas the other half had a single mutation (18 of 38 patients; 47%). We did not identify patients with more than two mutations in *CEBPA*. Nineteen of the 20 patients with two *CEBPA* mutations had both an N-terminal truncation mutation resulting in p30 CEBPA and a C-terminal missense mutation affecting the bZIP domain of CEBPA. One patient had a combination of an N-terminal in-frame deletion just 5′ to the second

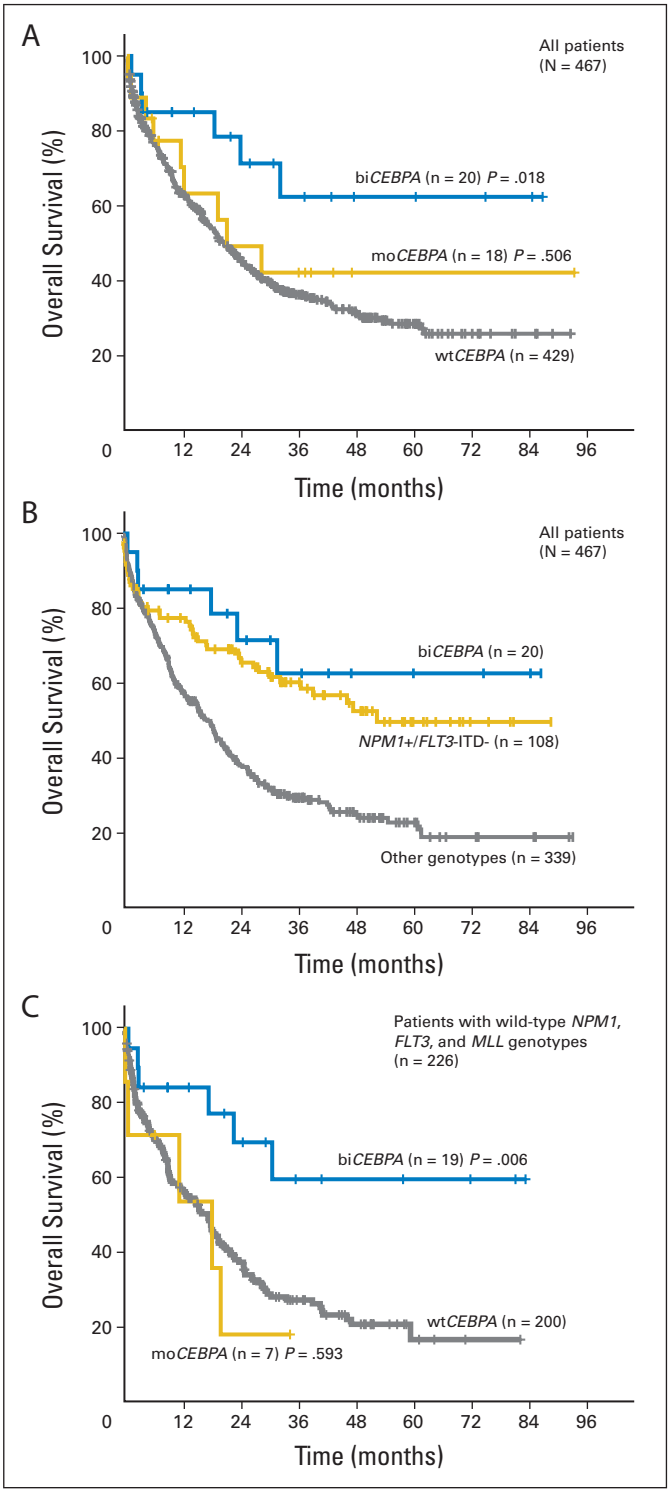

Fig 1. Overall survival according to the *CEBPA* mutational status in (A) all patients (N = 467); (B) patients with biallelic *CEBPA* mutations (bi*CEBPA*), patients with mutated *NPM1* without internal tandem duplication of the *FLT3* gene (*FLT3*-ITD; *NPM1*+/*FLT3*-ITD–), and patients with other genotypes; (C) and patients with no mutation in *NPM1*, *FLT3*, or *MLL* (n = 226). *P* values were determined using the log-rank test for pairwise comparison with wild-type (wt) *CEBPA*. mo*CEBPA*, monoallelic *CEBPA* mutation.

ATG and a C-terminal nonsense mutation in the transcriptional activation domain 2 (TAD2) resulting in a premature truncation of the CEBPA protein in TAD2. In all 20 patients with two *CEBPA* mutations, the distribution of the mutations was biallelic as determined by cloning analysis.[4] The 18 patients with only one heterozygous *CEBPA* mutation had predominately frameshift mutations, of which 13 were N-terminal truncation mutations resulting in p30 CEBPA. One patient had a frameshift mutation in the bZIP region, and two patients had frameshift mutations in the region between TAD2 and bZIP. Two patients had C-terminal missense mutations.

According to the number of mutated alleles in the *CEBPA* gene, we divided our patient cohort into the following three groups: patients with biallelic N- and C-terminal *CEBPA* mutations (bi*CEBPA*, n = 20); patients with a mo*CEBPA* mutation (n = 18); and patients without *CEBPA* gene mutations (wt*CEBPA*, n = 429).

*bi*CEBPA *Mutations, but Not mo*CEBPA *Mutations, Independently Predict a Favorable Outcome in Patients With CN-AML*

There was a balanced distribution among the three *CEBPA* subgroups with regard to random assignment, the number of induction cycles, the cumulative doses of cytarabine (Appendix Table A1), and the number of patients receiving allogeneic stem-cell transplantation (Table 1). Furthermore, we detected no significant differences among the three *CEBPA* subgroups with regard to age, WBC and platelet counts, percentage of bone marrow blasts, lactate dehydrogenase, or the number of patients with de novo, therapy-related, or secondary AML (Table 1). Whereas the bi*CEBPA* and wt*CEBPA* groups contained a similar ratio of male-to-female patients, there were significantly more female patients in the mo*CEBPA* group ($P = .006$). Patients with bi*CEBPA* had a significantly higher hemoglobin level ($P = .035$) and also a higher percentage of patients with French-American-British subtypes M1 or M2 ($P = .009$) compared with patients with mo*CEBPA* and wt*CEBPA* (Table 1).

Furthermore, we assessed whether the number of mutated *CEBPA* alleles had an impact on the prognosis. In accordance with previous studies, we found an improved median OS for all patients with a *CEBPA* mutation (n = 38) compared with patients with wt*CEBPA* (not reached *v* 20.4 months, respectively; $P = .028$; data not shown). Interestingly, only patients with bi*CEBPA* had a superior OS compared with patients with wt*CEBPA* (not reached *v* 20.4 months, respectively; $P = .018$), whereas patients with mo*CEBPA* had a similar OS as patients with wt*CEBPA* (20.9 *v* 20.4 months, respectively; $P = .506$; Fig 1A, Table 2). Consistent with these results, only patients with bi*CEBPA*, compared with patients with wt*CEBPA*, showed a trend toward a higher complete remission (CR) rate (85% *v* 66%, respectively; $P = .071$), lower relapse rate (41% *v* 63%, respectively; $P = .074$), and longer event-free survival (EFS; 16.2 *v* 8.5 months, respectively; $P = .064$). Relapse-free survival was similar in all subgroups (Table 2).

Most importantly, in a multivariable model, bi*CEBPA*, but not mo*CEBPA*, mutations were confirmed as an independent predictive variable for a favorable OS and EFS (Table 3). The adjusted hazard ratios (HRs) for OS (n = 369) were 0.285 (95% CI, 0.123 to 0.660; $P = .003$) for bi*CEBPA* and 0.751 (95% CI, 0.367 to 1.537; $P = .433$) for mo*CEBPA* compared with wt*CEBPA*. For EFS (n = 363), the adjusted HRs were 0.392 (95% CI, 0.196 to 0.784; $P = .008$) for bi*CEBPA* and 0.899 (95% CI, 0.486 to 1.665; $P = .736$) for mo*CEBPA*. The independent predictive value of bi*CEBPA* mutations on outcome was retained when censoring patients who underwent transplantation (OS: HR = 0.367, $P = .033$; EFS: HR = 0.367, $P = .005$).

The median follow-up time was 38.6 months for all patients and was not significantly different between patients with wt*CEBPA* (37.1 months), mo*CEBPA* (38.4 months), and bi*CEBPA* (39.1 months; $P = .829$). Furthermore, we compared the outcome of patients with bi*CEBPA* to the outcome of *NPM1*-mutated patients without *FLT3*-ITD (n = 108) because these patients represent the most common independent favorable prognostic subgroup in patients with CN-AML.[1] Patients with bi*CEBPA*, compared with patients with mutated *NPM1* without *FLT3*-ITD, showed a similar OS (not reached *v* 52.7 months, respectively; $P = .422$; Fig 1B) and EFS (16.2 *v* 30.1 months; $P = .926$; data not shown). In summary, the presence of a bi*CEBPA*, but not mo*CEBPA*, mutation was identified as an independent favorable prognostic factor in patients with CN-AML.

*bi*CEBPA *Mutations Were Never Found in Combination With Mutated* NPM1 *and Rarely Associated With* FLT3*-ITD*

In addition, we analyzed whether patients with bi*CEBPA* and mo*CEBPA* had a different frequency of mutations in the *NPM1*, *MLL*, and *FLT3* genes. Whereas only one (5%) of 20 patients with bi*CEBPA* had an additional *FLT3*-ITD, 11 (61%) of 18 patients with mo*CEBPA*

Table 2. Clinical Outcome of CN-AML Patients According to the No. of Mutated Alleles in *CEBPA*

End Point	wt*CEBPA* (n = 429)	mo*CEBPA* (n = 18)	bi*CEBPA* (n = 20)	*P** (wt*CEBPA v* mo*CEBPA*)	*P** (wt*CEBPA v* bi*CEBPA*)
Complete remission					
No. of patients/total	281/429	12/18	17/20		
Rate, %	66	67	85	.919	.071
Relapse					
No. of patients/total	176/281	7/12	7/17		
Rate, %	63	58	41	.751	.074
Median OS, months	20.4	20.9	NR	.506	.018
Median EFS, months	8.5	6.5	16.2	.771	.064
Median RFS, months	16.7	27.5	NR	.685	.209

Abbreviations: CN-AML, cytogenetically normal acute myeloid leukemia; wt*CEBPA*, wild-type *CEBPA*; mo*CEBPA*, monoallelic *CEBPA* mutation; bi*CEBPA*, biallelic *CEBPA* mutations; OS, overall survival; NR, not reached; EFS, event-free survival; RFS, relapse-free survival.
**P* values were calculated using the Mann-Whitney *U* test.

Table 3. Multiple Cox Regression Analysis of mo*CEBPA* and bi*CEBPA* As Prognostic Marker for OS and EFS in CN-AML

Variable in Final Models	OS			EFS		
	HR	95% CI	*P**	HR	95% CI	*P**
bi*CEBPA v* wt*CEBPA*	0.29	0.12 to 0.66	.003	0.39	0.20 to 0.78	.008
mo*CEBPA v* wt*CEBPA*	0.75	0.37 to 1.54	.433	0.90	0.49 to 1.67	.736
NPM1 mutation *v* no *NPM1* mutation	0.34	0.22 to 0.51	< .001	0.31	0.22 to 0.45	< .001
FLT3-ITD mutation *v* no *FLT3*-ITD mutation	0.76	0.46 to 1.26	.281	0.74	0.46 to 1.18	.207
Interaction term *NPM1*/*FLT3*-ITD†	1.66	0.84 to 3.26	.144	1.73	0.94 to 3.20	.079
Age (10-year increase)	1.35	1.20 to 1.53	< .001	1.18	1.07 to 1.31	.002
Sex	0.92	0.69 to 1.21	.542	0.93	0.72 to 1.19	.568
De novo AML *v* non de novo AML	0.86	0.60 to 1.24	.428	0.99	0.71 to 1.38	.971
FAB: M1/M2 *v* M0, M3, M4, M5, M6	1.20	0.89 to 1.62	.236	1.14	0.87 to 1.50	.355
WBC count, $\times 10^9$/L (10-fold value)	1.48	1.09 to 1.99	.011	1.40	1.06 to 1.83	.016
Platelet count, $\times 10^9$/L (10-fold value)	0.78	0.53 to 1.14	.198	0.73	0.51 to 1.04	.084
Hemoglobin, g/L	1.00	0.99 to 1.01	.839	1.00	1.00 to 1.01	.355
Bone marrow blasts, %	1.00	1.00 to 1.01	.654	1.00	1.00 to 1.01	.385
LDH, U/L (10-fold value)	1.91	0.98 to 3.74	.058	1.32	0.71 to 2.45	.374

NOTE. To evaluate the independent prognostic impact of bi*CEBPA* on outcome, all candidate prognostic factors were included in the Cox regression model without selection of variables. *FLT3*-TKD and *MLL*-PTD mutations, both not showing a significant effect on outcome either in univariate or multivariate analyses, have not been added to the model to maintain a high number of patients with complete data set. The analyses were performed using 369 complete patients with regard to OS and 363 complete patients with regard to EFS for the candidate prognostic factors. For sensitivity analyses, we also included *FLT3*-TKD and *MLL*-PTD and evaluated a modified OS and EFS in multiple Cox regression, censoring time to event at the date of allogeneic transplantation, if performed. These analyses revealed qualitatively identical results. Censoring allogeneic transplantation, the adjusted HRs for OS were 1.068 (95% CI, 0.494 to 2.309; $P = .868$) for mo*CEBPA* and 0.367 (95% CI, 0.146 to 0.921; $P = .033$) for bi*CEBPA* compared with wt*CEBPA*. For EFS, the adjusted HRs were 1.223 (95% CI, 0.661 to 2.262; $P = .521$) for mo*CEBPA* and 0.367 (95% CI, 0.183 to 0.735; $P = .005$) for bi*CEBPA*.

Abbreviations: mo*CEBPA*, monoallelic *CEBPA* mutation; bi*CEBPA*, biallelic *CEBPA* mutations; OS, overall survival; EFS, event-free survival; CN-AML, cytogenetically normal acute myeloid leukemia; HR, hazard ratio; wt*CEBPA*, wild-type *CEBPA*; *NPM1*, nucleophosmin; *FLT3*-ITD, internal tandem duplication of the *FLT3* gene; AML, acute myeloid leukemia; FAB, Frensh-American-British; LDH, lactate dehydrogenase.

*P values were calculated using the Wald test.

†Interaction term *NPM1*/*FLT3*-ITD (*NPM1* positive/*FLT3*-ITD positive = 1; other *NPM1*/*FLT3*-ITD combinations = 0).

had at least one additional aberration (Fig 2). Four patients with mo*CEBPA* had one additional mutation; two had an *NPM1* mutation, one had an *FLT3*-ITD, and one had an *MLL*-PTD. Seven patients with mo*CEBPA* had two additional aberrations: six had mutated *NPM1* and an *FLT3*-ITD, and one had an *FLT3*-TKD and an *FLT3*-ITD (Fig 2). We found statistically significant differences in the frequency of *NPM1* mutations ($P = .001$) and *FLT3*-ITD ($P = .014$) among the three subgroups (Table 1). Whereas frequencies of *MLL*-PTD and *FLT3*-TKD were not different, patients with bi*CEBPA* were never associated with mutated *NPM1*, which was statistically significant compared with mo*CEBPA* (0% *v* 41%, respectively; $P = .002$; Table 1) and wt*CEBPA* (0% *v* 43%, respectively; $P < .001$). Furthermore, bi*CEBPA* mutations were rarely associated with *FLT3*-ITD (5%) compared with mo*CEBPA* (44%; $P = .007$) and wt*CEBPA* (23%; $P = .059$). By contrast, patients with mo*CEBPA* had a significantly higher frequency of additional *FLT3*-ITD compared with patients with wt*CEBPA* (44% *v* 23%, respectively; $P = .037$) or patients with bi*CEBPA* (44% *v* 5%, respectively; $P = .007$). The percentage of *NPM1* mutations was not different in patients with mo*CEBPA* and wt*CEBPA* (41% *v* 43%, respectively; $P = .869$; Table 1).

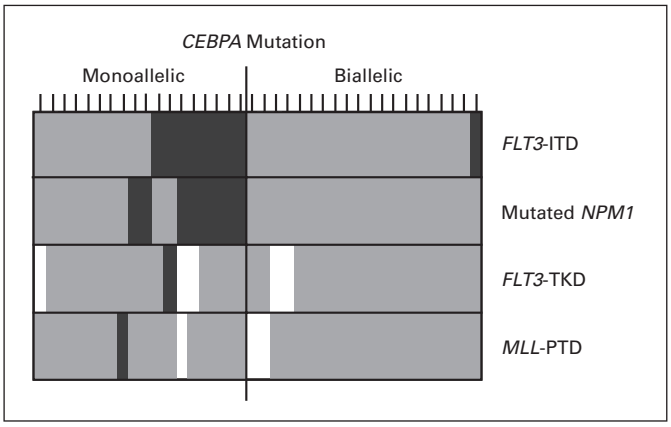

Fig 2. Frequencies of genetic aberrations in patients with monoallelic *CEBPA* mutation and biallelic *CEBPA* mutations. Each tick mark at the top of the figure indicates one patient, and patients who are positive for the respective mutation are marked in black. Missing information is indicated as a white space. *FLT3*-ITD, internal tandem duplication of the *FLT3* gene; *FLT3*-TKD, tyrosine kinase domain mutations; *MLL*-PTD, partial tandem duplication of the *MLL* gene.

To minimize confounding effects of *NPM1*, *FLT3*, and *MLL* mutations on the survival in the three groups (bi*CEBPA*, mo*CEBPA*, and wt*CEBPA*), we analyzed only patients without additional mutations in the *NPM1*, *MLL*, or *FLT3* genes (n = 226). In this comparison, the 19 patients with only bi*CEBPA* retained a statistically better median OS compared with patients with wt*CEBPA* without additional mutations in *NPM1*, *MLL*, or *FLT3* (n = 200; not reached *v* 18.1 months, respectively; $P = .006$), whereas the seven patients with mo*CEBPA* without additional mutations had no different OS compared with patients with wt*CEBPA* without additional mutations in *NPM1*, *MLL*, or *FLT3* (19.0 *v* 18.1 months, respectively; $P = .593$; Fig 1C). These results indicate that patients with bi*CEBPA* have a lower association with additional *NPM1* and *FLT3*-ITD mutations and that the favorable outcome in these patients is not a result of the lack of additional mutations in *NPM1*, *MLL*, or *FLT3*.

*AML Blasts From Patients With bi*CEBPA *Mutations Show a Characteristic Gene Expression Pattern*

To explore the impact of bi*CEBPA* on gene expression, the gene expression profiles of 61 patients with CN-AML (seven patients with bi*CEBPA*, eight patients with mo*CEBPA*, and 46 patients with wt-*CEBPA*) were analyzed. In an initial step, we identified a total of 83 genes that were differentially expressed between bi*CEBPA* and wt-*CEBPA* patients (Appendix Table A2, online only). According to the applied selection criteria, less than one false-positive gene is expected within this list. Comparison of gene expression between bi*CEBPA* and mo*CEBPA* AML was hampered by the small number of patients in both groups. However, using a significance level of $\alpha = .05$ and a fold change of more than 1.5 as significance criteria, 82 differentially regulated genes were identified (Appendix Table A3, online only). Thirty-three of these genes overlapped with the genes identified in the bi*CEBPA* versus wt*CEBPA* comparison ($P < .0001$; Appendix Fig A1, online only). Because they were selected by highly stringent significance criteria, these 33 genes likely represent true positives. Genes significantly downregulated in patients with bi*CEBPA*, compared with patients with mo*CEBPA* and wt*CEBPA* AML, include multiple members of the homeobox gene family (*HOXA5*, *HOXA9*, *HOXA10*, *HOXB2*, and *HOXB6*), *CD34*, and lymphoid markers *CD9*, *CD52*, and *TSPO*.

A more detailed analysis of differentially expressed genes revealed that, in general, the gene expression profiles were more homogeneous among patients with bi*CEBPA* than among patients with mo*CEBPA*. For example, all patients with bi*CEBPA* uniformly showed a strong downregulation of the *HOXB2* and *HOXA9* genes, whereas in mo-*CEBPA* patients, a wider range of *HOXB2* and *HOXA9* expression values was observed (Figs 3A and 3B). The two patients within the mo*CEBPA* group who also had an *NPM1* mutation showed the highest *HOXB2* and *HOXA9* expression levels. Similar results were observed for the other *HOX* cluster genes (*HOXA5*, *HOXA10*, and *HOXB6*; data not shown), as well as for several other differentially regulated genes including *CD52*, *CD34*, *CLC* (Charcot-Leyden crystal protein), and *S100B* (Figs 3C to 3F). These results indicate that patients with bi*CEBPA* show a more prominent deregulation of genes with known importance in AML pathogenesis than patients with mo*CEBPA*.

DISCUSSION

Using a large cohort of genetically well-defined and homogeneously treated patients with CN-AML, we were able to show that bi*CEBPA* mutations define a distinct clinical and molecular subtype of AML (Tables 1 and 2). Our findings suggest that the favorable clinical outcome that has been reported for patients with all *CEBPA* mutations in previous studies is in fact a result of the subgroup of patients with bi*CEBPA* mutations, which results in a disruption of the N terminus and C terminus of the CEBPA protein (Fig 1, Table 2). Our data are supported by two recent studies in patients with AML that showed that two *CEBPA* mutations are required for a favorable prognosis.[19,20] We illustrate that only when both alleles in *CEBPA* are affected, patients with CN-AML have a similarly favorable prognosis as patients with mutant *NPM1* without *FLT3*-ITD (Fig 1B). Moreover, only a biallelic disruption, not a monoallelic disruption, of *CEBPA* emerged as an independent prognostic factor predicting a favorable outcome in patients with CN-AML (Table 3). The improved outcome of patients with bi*CEBPA* might be a consequence of a higher CR rate and a lower relapse rate. However, a larger analysis will be required to demonstrate significant differences for the CR rate and consequently for relapse-free survival.

Both *CEBPA* and *NPM1* mutations have been categorized as potential type II mutations according to the two-step model of leukemogenesis.[21] In accordance with this model, our data suggest that bi*CEBPA* and *NPM1* mutations are mutually exclusive in patients with CN-AML.

We found that patients with bi*CEBPA* had a low frequency of additional gene mutations (ie, *NPM1*, *FLT3*, and *MLL*; Fig 2). Accordingly, among *CEBPA*-mutated patients with AML, additional *FLT3*-ITD and *NPM1* mutations were exclusively identified in patients with single *CEBPA* mutations.[20] Interestingly, there is evidence in patients with familiar *CEBPA* mutations that the C-terminal CEBPA mutations are secondary events. In all reported patients with familiar *CEBPA* mutations, the patients had a germline N-terminal truncation mutation and acquired a second C-terminal CEBPA mutation at AML diagnosis.[22-25] These studies indicate that N- and C-terminal CEBPA mutations might cooperate to induce AML. In transient transfection studies, the N- and C-terminal CEBPA mutations strongly synergize in the inhibition of transactivation of the CEBPA-responsive G-CSFR promoter.[20] Therefore, bi*CEBPA* mutations might represent two separate hits in the process of leukemogenesis.

Furthermore, we showed that patients with a mo*CEBPA* mutation have a higher incidence of *FLT3*-ITD mutations than patients with wt*CEBPA* or bi*CEBPA* (Fig 2, Table 1). Studies on samples of patients with relapsed AML suggest that *CEBPA* mutations are a primary event in leukemogenesis,[26,27] whereas *FLT3*-ITD mutations are proposed to be a secondary event, important for disease progression.[28,29] So far, there are contradictory studies concerning whether a coexisting *FLT3*-ITD adversely affects the favorable prognosis of patients with *CEBPA* mutations.[6,30,31] To exclude that the different clinical outcome in patients with mo*CEBPA* versus bi*CEBPA* is a result of the different frequencies of additional gene mutations, we separately analyzed the outcome of patients without additional *NPM1*, *FLT3*, or *MLL* mutations and were able to show that the better OS of patients with bi*CEBPA* was independent of mutations in the *NPM1*, *MLL*, or *FLT3* gene (Fig 2C).

Along with the different spectrum of cooperating mutations, we also observed differences between the gene expression profiles of patients with bi*CEBPA* and mo*CEBPA*. We indentified 33 genes that were differentially expressed in patients with bi*CEBPA* compared with patients with wt*CEBPA* and mo*CEBPA* (Appendix Fig A1 and Appendix Table A3), 29 of which have previously been associated with mutant *CEBPA*.[32,33] Compared with patients with bi*CEBPA* mutations, who showed a relatively homogeneous pattern of gene deregulation including strong and uniform downregulation of several *HOX* cluster genes, patients with mo*CEBPA* showed greater variability in their gene expression profiles. Interestingly, patients with mo*CEBPA* with mutant *NPM1* showed the highest levels of *HOX* gene expression within our cohort. *NPM1* mutations have previously been associated with *HOX* gene overexpression,[34] and low levels of *HOX* gene expression have been associated with favorable clinical outcomes independent of the cytogenetic subgroup.[32,33] In line with the greater variability of gene expression profiles observed in patients with mo-*CEBPA*, it has recently been shown that by using a microarray-based

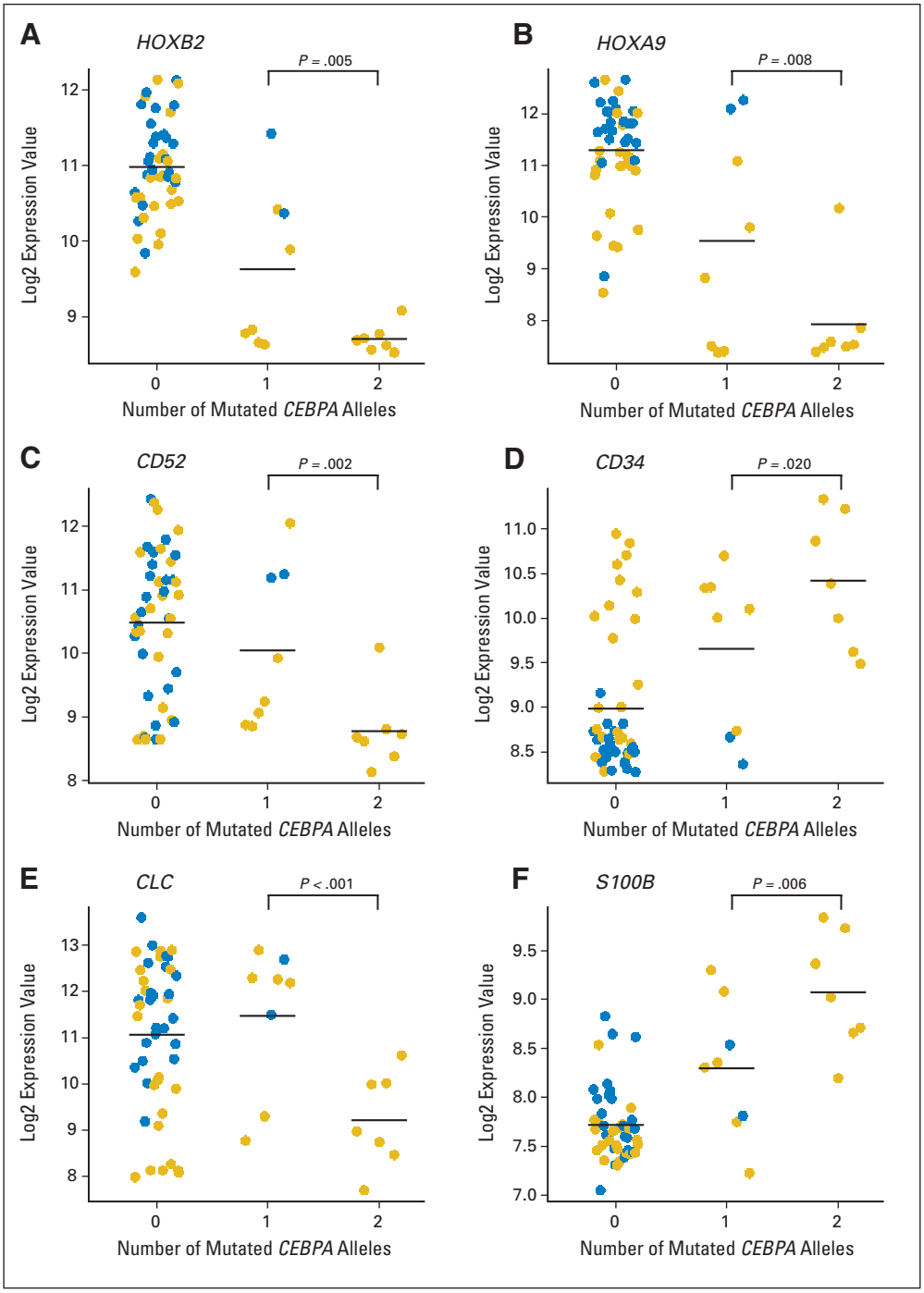

Fig 3. Dot plots showing the logarithmic expression of selected genes according to the *CEBPA* mutational status. Blue circles represent patients with a mutation in *NPM1*, and gold circles represent patients with wild-type *NPM1*. The horizontal line marks the mean expression in each group. *P* values were determined using the *Z* test statistic (Twilight software package; Max Planck Institute for Molecular Genetics, Berlin, Germany) for comparison between monoallelic *CEBPA* mutation and biallelic *CEBPA* mutations. (A) *HOXB2*; (B) *HOXA9*; (C) *CD52*; (D) *CD34*; (E) *CLC*; (F) *S100B*.

class prediction algorithm, only double *CEBPA* mutations could be correctly predicted, whereas patients with single *CEBPA* mutations were frequently misclassified.[35] Although based on a limited number of patients, our data illustrate that on the level of gene expression, patients with bi*CEBPA* are a relatively homogeneous group, whereas the greater heterogeneity among patients with mo*CEBPA* mutations may partly be related to the influence of cooperating mutations.

In summary, we have shown here, in a large uniformly treated cohort of patients with CN-AML, that *CEBPA* mutant patients with AML represent a heterogeneous group that can further be separated according to the number of mutated alleles in the *CEBPA* gene. These data provide deeper insight into the biology of *CEBPA*-mutated AML and will have a major impact on diagnostic procedures and patient stratification for risk-adapted therapies.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

The author(s) indicated no potential conflicts of interest.

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