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ORIGINAL REPORT

Prognostic Significance of, and Gene and MicroRNA Expression Signatures Associated With, *CEBPA* Mutations in Cytogenetically Normal Acute Myeloid Leukemia With High-Risk Molecular Features: A Cancer and Leukemia Group B Study

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ABSTRACT

Purpose
To evaluate the prognostic significance of *CEBPA* mutations in the context of established molecular markers in cytogenetically normal (CN) acute myeloid leukemia (AML) and gain biologic insights into leukemogenesis of the CN-AML molecular high-risk subset (*FLT3* internal tandem duplication [ITD] positive and/or *NPM1* wild type) that has a significantly higher incidence of *CEBPA* mutations than the molecular low-risk subset (*FLT3*-ITD negative and *NPM1* mutated).

Patients and Methods
One hundred seventy-five adults age less than 60 years with untreated primary CN-AML were screened before treatment for *CEBPA*, *FLT3*, *MLL*, *WT1*, and *NPM1* mutations and *BAALC* and *ERG* expression levels. Gene and microRNA (miRNA) expression profiles were obtained for the CN-AML molecular high-risk patients.

Results
CEBPA mutations predicted better event-free ($P = .007$), disease-free ($P = .014$), and overall survival ($P < .001$) independently of other molecular and clinical prognosticators. Among patients with *CEBPA* mutations, 91% were in the CN-AML molecular high-risk group. Within this group, *CEBPA* mutations predicted better event-free ($P < .001$), disease-free ($P = .004$), and overall survival ($P = .009$) independently of other molecular and clinical characteristics and were associated with unique gene and miRNA expression profiles. The major features of these profiles were upregulation of genes (eg, *GATA1*, *ZFPM1*, *EPOR*, and *GFI1B*) and miRNAs (ie, the *miR-181* family) involved in erythroid differentiation and downregulation of homeobox genes.

Conclusion
Pretreatment testing for *CEBPA* mutations identifies CN-AML patients with different outcomes, particularly in the molecular high-risk group, thus improving molecular risk-based classification of this large cytogenetic subset of AML. The gene and miRNA expression profiling provided insights into leukemogenesis of the CN-AML molecular high-risk group, indicating that *CEBPA* mutations are associated with partial erythroid differentiation.

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INTRODUCTION

The broad range of disease-free survival (DFS) and overall survival (OS) durations reported in cytogenetically normal (CN) acute myeloid leukemia (AML) patients can be largely explained by their genomic heterogeneity, with several molecular markers proven to be prognostic.[1] Among these, *FLT3* internal tandem duplication (*FLT3*-ITD),[2-4] *FLT3* tyrosine kinase domain,[5] and *WT1* mutations[6,7] and high *BAALC*[8-11] and *ERG*[12,13] expression levels have been validated as adverse prognostic markers, and *NPM1* mutations[14-17] have been validated as favorable prognostic markers. Patients with CN-AML and *NPM1* mutations and no *FLT3*-ITD (*NPM1* mutated/*FLT3*-ITD negative) are considered to comprise a molecular low-risk group because their outcome is superior to CN-AML patients

with *FLT3*-ITD (*FLT3*-ITD positive) and/or wild-type *NPM1*, who constitute a molecular high-risk group.[15-17] The former have a favorable outcome similar to that of patients with core binding factor AML.[18]

CEBPA encodes a protein member of the basic region leucine zipper (bZIP) transcription factor family that is essential for myeloid differentiation.[19] *CEBPA* mutations occur predominantly in CN-AML,[20,21] having been reported in 13% to 19% of such patients.[10,20,22,23] There are two main categories of *CEBPA* mutations. N-terminal nonsense mutations prevent expression of the full-length protein and result in a truncated isoform with dominant-negative activity, whereas the C-terminal mutations occurring in the bZIP domains are usually in-frame and encode mutant proteins lacking DNA binding and/or homodimerization activities.[19,20] Both mutation types are predicted to confer loss of function in the C/EBPα protein.

Whereas initial reports focused on the prognostic significance of *CEBPA* mutations in the intermediate-risk cytogenetic group of AML patients,[24,25] other studies[10,22,23] have shown the favorable impact of *CEBPA* mutations on clinical outcome in CN-AML. The predictive value of *CEBPA* mutations has been studied concurrently with *FLT3*-ITD and *MLL* partial tandem duplication (*MLL*-PTD),[22] *FLT3*-ITD and *BAALC* expression,[10] and *FLT3*-ITD and *NPM1* mutations.[23] However, no report described the clinical significance of *CEBPA* mutations in the context of all established prognostic molecular markers in CN-AML, including *WT1* mutations and *ERG* overexpression. Therefore, we tested the predictive value of *CEBPA* mutations in a relatively large group of CN-AML patients concurrently with the aforementioned prognostic markers. To gain biologic insights regarding the role of *CEBPA* mutations in the CN-AML molecular high-risk patients, who harbor *CEBPA* mutations significantly more often than molecular low-risk patients, we derived *CEBPA* mutation-associated gene and microRNA (miRNA) expression profiles.

PATIENTS AND METHODS

Patients, Treatment, Cytogenetic, and Molecular Analyses

CEBPA mutations were analyzed in 175 adults younger than 60 years of age with untreated, primary CN-AML enrolled onto the Cancer and Leukemia Group B (CALGB) treatment protocols 9621[26] and 19808[27] (see Appendix, online only). Pretreatment cytogenetic analyses of bone marrow were performed by CALGB-approved institutional cytogenetic laboratories as part of CALGB 8461, a prospective cytogenetic companion study, and were centrally reviewed, as previously reported.[28] To be considered CN, $\geq$ 20 metaphase cells had to be analyzed, and the karyotype had to be found to be normal. The presence or absence of the *CEBPA* mutations (see Appendix),[22] *FLT3*-ITD,[4] *FLT3* tyrosine kinase domain mutations,[4,5,29] *MLL*-PTD,[30,31] *NPM1* mutations,[15] *WT1* mutations,[6] and *BAALC*[8] and *ERG*[13] expression levels was determined centrally in pretreatment samples as described previously. Written informed consent for these studies was obtained from all patients.

Gene and miRNA Expression Profiling

Suitable RNA samples from 107 patients in the CN-AML molecular high-risk group were analyzed using Affymetrix U133 plus 2.0 GeneChips (Affymetrix, Santa Clara, CA), as previously reported.[5,32] Suitable RNA samples from 64 patients in the CN-AML molecular high-risk group enrolled onto CALGB 19808 were analyzed for miRNA expression using a previously reported Ohio State University customized miRNA chip.[33] Images of the gene and miRNA microarrays were acquired, and calculation, normalization, and filtering of signal intensity for each microarray spot were performed as described in the Appendix.

Statistical Methods

Definitions of clinical end points (ie, complete remission [CR], event-free survival [EFS], DFS, and OS) are provided in the Appendix. Pretreatment clinical features of patients with and without *CEBPA* mutations were compared using Fisher's two-sided exact and Wilcoxon rank sum tests for categoric and continuous variables, respectively. Estimated probabilities of EFS, DFS, and OS were calculated using the Kaplan-Meier method, and the log-rank test evaluated differences between survival distributions. Proportional hazards models were constructed for survival end points, using a limited backwards elimination procedure (see footnotes to Table 3 for factors examined for model inclusion). Variables remaining in the final models were those significant at $\alpha = .05$ and those important molecular variables that were confounded with the main variable, *CEBPA* mutation status. The proportional hazards assumption was checked for each variable individually. If the proportional hazards assumption was not met for a particular variable, then an artificial time-dependent covariate was included in all models containing that variable.[34]

For gene expression analysis, a filtering step was performed to remove probe sets that displayed low variation in expression across arrays (see Appendix). A comparison of expression of the 23,204 filtered probe sets between patients with (n = 26) and without (n = 81) *CEBPA* mutations was performed in CN-AML molecular high-risk patients, making an adjustment for protocol. A univariable significance level of $\alpha = .001$ was used, resulting in approximately 23 expected false-positive probe sets assuming no gene expression differences between the two groups.

For miRNA expression analysis, a comparison of expression of the 305 filtered human miRNA probes between patients with (n = 18) and without (n = 46) *CEBPA* mutations was performed in patients with molecular high-risk features, making an adjustment for the batch in which arrays were hybridized. A univariable significance level of $\alpha = .005$ was used, resulting in approximately one or two expected false-positive probes assuming no miRNA expression differences between the two groups.

Microarray gene and miRNA expression analyses were performed using BRB-ArrayTools version 3.4.0 (R. Simon and A.P. Lam, National Cancer Institute, Bethesda, MD) and using the R version 2.3.1 (R Foundation for Statistical Computing, Vienna, Austria). All analyses were performed by the CALGB Statistical Center.

RESULTS

CEBPA *Mutations As Predictors of Outcome in AML*

Among 175 patients analyzed, 143 (82%) had only *CEBPA* wild-type alleles (*CEBPA*wt), whereas 32 (18%) harbored *CEBPA* mutations. Twenty-one patients had N-terminal mutations (seven alone and 14 concurrent with a C-terminal mutation), and 11 patients had C-terminal mutations only. Four patients had an insertion of six nucleotides predicting for a histidine-proline duplication in the transactivation domain 2 of the encoded protein (*CEBPA*$^{\text{HP196-197ins}}$) as the only change. Although *CEBPA*$^{\text{HP196-197ins}}$ was previously considered to be a *CEBPA* mutation,[22] it has recently been reported to be a germline polymorphism of the *CEBPA* gene.[35-38] Therefore, we included *CEBPA*$^{\text{HP196-197ins}}$ patients in the *CEBPA*wt subset.

At diagnosis, patients with *CEBPA* mutations had significantly higher hemoglobin levels ($P = .02$), lower platelet counts ($P = .009$), and lower incidence of extramedullary disease ($P = .03$) compared with *CEBPA*wt patients (Table 1). Molecularly, patients with *CEBPA* mutations were more likely to lack *NPM1* mutations ($P < .0001$) and to be in the molecular high-risk ($P < .001$) and high *BAALC* expression ($P = .003$) groups than *CEBPA*wt patients. Other pretreatment characteristics (Table 1) and the proportion of patients receiving autologous stem-cell transplantation (88% for mutated *CEBPA* *v* 82% for *CEBPA*wt; $P = .76$) did not differ between the groups.

Table 1. Clinical and Molecular Characteristics According to *CEBPA* Mutational Status in Cytogenetically Normal Acute Myeloid Leukemia Patients

Characteristic	Mutated *CEBPA* (n = 32)		Wild-Type *CEBPA* (n = 143)		*P*
	No. of Patients	%	No. of Patients	%	
Age, years					.69
Median	44		46		
Range	19-59		18-59		
Male	18	56	67	47	.43
Race					1.00
White	29	91	126	89	
Nonwhite	3	9	16	11	
Hemoglobin, g/dL					.02
Median	10.1		9.4		
Range	4.9-13.4		4.8-13.6		
Platelet count, $\times 10^9$/L					.009
Median	38		61		
Range	7-232		11-445		
WBC, $\times 10^9$/L					.17
Median	19.1		30.2		
Range	4.9-295.0		1.4-273.0		
% of blood blasts					.07
Median	64.5		58		
Range	10-97		0-95		
% of bone marrow blasts					.15
Median	61.5		70		
Range	26-98		10-99		
Extramedullary disease	5	16	50	35	.03
FLT3-ITD					.07
Negative	24	75	81	57	
Positive	8	25	62	43	
NPM1					< .0001
Wild type	26	81	32	22	
Mutated	6	19	111	78	
Molecular risk group*					< .001
Low risk	3	9	57	40	
High risk	29	91	86	60	
FLT3-TKD					.20
Negative	31	97	125	88	
Positive	1	3	17	12	
MLL-PTD					.26
Negative	28	88	134	94	
Positive	4	12	9	6	
WT1					.25
Wild type	26	81	119	89	
Mutated	6	19	15	11	
BAALC expression†					.003
Low	7	24	58	55	
High	22	76	47	45	
Unkown	3		38		
ERG expression‡					.26
Low	19	73	62	59	
High	7	27	43	41	
Unknown	6		38		

Abbreviations: *FLT3*-ITD, internal tandem duplication of the *FLT3* gene; *FLT3*-TKD, tyrosine kinase domain mutations of the *FLT3* gene; *MLL*-PTD, partial tandem duplication of the *MLL* gene.

*Molecular low-risk group is defined by the absence of *FLT3*-ITD and presence of *NPM1* mutation. Molecular high-risk group is defined by the presence of *FLT3*-ITD and/or the lack of *NPM1* mutation.

†*BAALC* expression values were dichotomized at the median to define high and low expressers.

‡*ERG* expression values were dichotomized at the median (Cancer and Leukemia Group B 19808 trial)[13] or at the 75th percentile (Cancer and Leukemia Group B 9621 trial)[12] to define high and low expressers.

With a median follow-up time of 4.8 years (range, 2.7 to 9.9 years) for patients with no events (ie, failure to achieve CR, relapse, or death), patients with *CEBPA* mutations had a significantly better EFS than *CEBPA*wt patients ($P = .017$); 5-year EFS rates were 53% and 30%, respectively (Fig 1A; Table 2). Patients with *CEBPA* mutations had a trend for better CR rates ($P = .12$), DFS ($P = .075$), and OS ($P = .10$) compared with *CEBPA*wt patients (Table 2). In multivariable analyses, *CEBPA* mutations were independently associated with

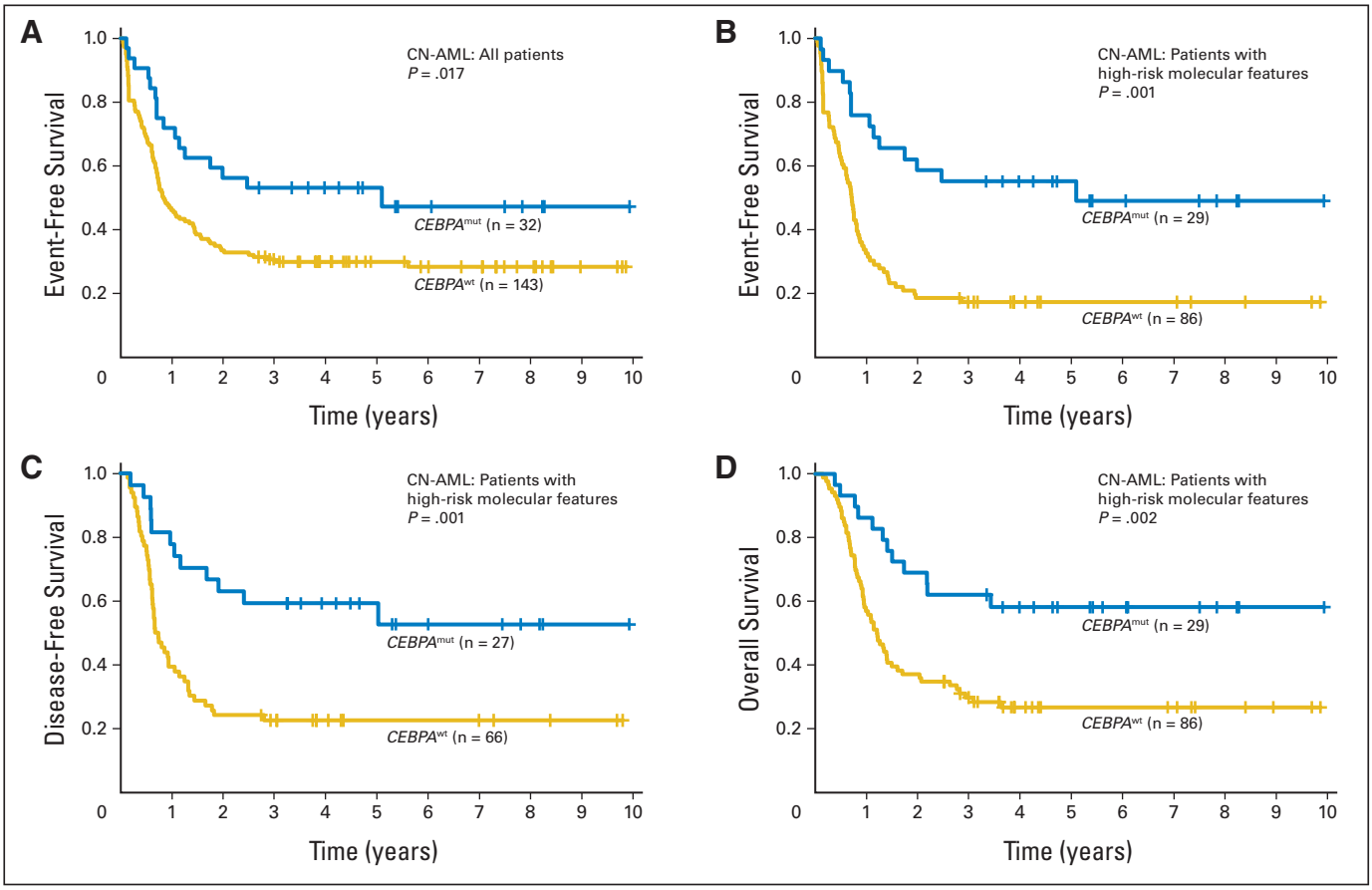

Fig 1. Outcome of cytogenetically normal acute myeloid leukemia (CN-AML) according to *CEBPA* mutational status. (A) Event-free survival of all patients with CN-AML. (B) Event-free survival of patients with molecular high-risk CN-AML (ie, patients with *FLT3* internal tandem duplication and/or wild-type *NPM1*). (C) Disease-free survival of patients with molecular high-risk CN-AML. (D) Overall survival of patients with molecular high-risk CN-AML. *CEBPA*^{mut}, patients with *CEBPA* mutations; *CEBPA*^{wt}, patients with wild-type *CEBPA*.

better EFS ($P = .007$), DFS ($P = .014$), and OS ($P < .001$) after adjusting for other molecular and clinical variables (Table 3). Compared with patients with *CEBPA*wt, patients with *CEBPA* mutations had a 60% reduction of the risk for failure to achieve CR, relapse, or death. A multivariable model could not be constructed for CR because most patients (30 of 32 patients) with *CEBPA* mutations achieved CR.

Because 29 (91%) of 32 patients with *CEBPA* mutations were in the CN-AML molecular high-risk group, we focused subsequent outcome analyses on this subset. Only three patients in the molecular low-risk group harbored *CEBPA* mutations; thus, we were unable to evaluate the prognostic significance of *CEBPA* mutations in this group. Akin to the overall group, in the molecular high-risk subset, patients with *CEBPA* mutations had lower platelet counts ($P = .003$) and a trend for higher hemoglobin levels ($P = .09$; Appendix Table A1, online only). They were also less likely to be *FLT3*-ITD positive ($P < .0001$) and harbor *NPM1* mutations ($P < .0001$) than *CEBPA*wt patients (Appendix Table A1). Frequencies and distribution of mutations and gene expression according to *CEBPA* mutation status are shown in Appendix Figure A1.

With a median follow-up time of 4.5 years (range, 2.8 to 9.9 years) for patients with no events, patients with *CEBPA* mutations had better outcomes. Patients with *CEBPA* mutations, compared with *CEBPA*wt patients, had a trend for a better CR rate (93% *v* 77%, respectively; $P = .06$) and significantly greater 5-year rates of EFS (55% *v* 17%, respectively; $P < .001$), DFS (53% *v* 23%, respectively; $P = .001$), and OS (58% *v* 27%, respectively; $P = .002$; Fig 1B to 1D; Table 2). In a multivariable analysis, *CEBPA* mutations independently predicted longer EFS ($P < .001$), after adjusting for *WT1* mutational status ($P = .03$), hemoglobin level ($P = .04$), and WBC count ($P = .03$; Table 3). *CEBPA* mutations also independently predicted longer DFS ($P = .004$), after adjusting for *FLT3*-ITD status ($P = .008$) and hemoglobin level ($P = .003$), and longer OS ($P = .009$), after adjusting for *FLT3*-ITD ($P = .009$) and *WT1* ($P = .002$) mutational status (Table 3). *CEBPA* mutations conferred 60% to 70% reduction in the risk of an event compared with *CEBPA*wt (Table 3). A multivariable model could not be constructed for CR because most patients with *CEBPA* mutations (27 of 29 patients) achieved CR.

Gene Expression Profiling

To gain insight into the biologic role of *CEBPA* mutations in the CN-AML molecular high-risk group, we performed both gene and miRNA microarray analyses. A gene expression signature consisting of 2,475 probe sets was associated with *CEBPA* mutational status. Of the differentially expressed probe sets, approximately 40% were overexpressed in patients with *CEBPA* mutations. In addition to overexpression of *CD34*, *CD38*, and *CD7*, a prominent characteristic of

Table 2. Outcomes of Cytogenetically Normal Acute Myeloid Leukemia Patients According to *CEBPA* Mutational Status

End Point	Mutated *CEBPA*	Wild-Type *CEBPA*	*P*
All patients			
No. of patients	32	143	
Complete remission			.12
No. of patients	30	115	
%	94	80	
Event-free survival			.017
Median, years	5.1	0.8	
5-Year rate, %	53	30	
95% CI	35 to 69	23 to 38	
Disease-free survival			.075
Median, years	Not reached	1.3	
5-Year rate, %	50	37	
95% CI	30 to 68	28 to 46	
Overall survival			.10
Median, years	Not reached	1.9	
5-Year rate, %	56	41	
95% CI	37 to 71	33 to 49	
Molecular high-risk patients			
No. of patients	29	86	
Complete remission			.06
No. of patients	27	66	
%	93	77	
Event-free survival			< .001
Median, years	5.1	0.7	
5-Year rate, %	55	17	
95% CI	36 to 71	10 to 26	
Disease-free survival			.001
Median, years	Not reached	0.7	
5-Year rate, %	53	23	
95% CI	31 to 71	13 to 33	
Overall survival			.002
Median, years	Not reached	1.2	
5-Year rate, %	58	27	
95% CI	38 to 74	18 to 37	

this signature was the upregulation of genes involved in erythroid differentiation, including *GATA1*, *ZFPM1* (also known as *FOG1*), *HEMGN*, *EPOR*, *GFI1B*, *KLF1*, *ANK1*, *TFRC* (*CD71*), and genes encoding erythrocyte membrane proteins and hemoglobin chains in patients with *CEBPA* mutations (Fig 2). These findings were consistent with higher hemoglobin levels observed in patients with *CEBPA* mutations at diagnosis. In contrast, genes involved in myeloid differentiation, such as *RUNX1*, *SPI1* (also known as *PU.1*), and *ID1*, were downregulated in patients with *CEBPA* mutations, as were several members of the homeobox family (*HOXA1*, *HOXA2*, *HOXA3*, *HOXA4*, *HOXA6*, *HOXA5*, *HOXA7*, *HOXA9*, *HOXA10*, *HOXB2*, *HOXB3*, *HOXB4*, *HOXB5*, *HOXB6*, and *MEIS1*; Fig 2). In this subset of patients, genes involved in proliferation signaling pathways, such as *FLT3*, *LYN*, and members of the *RAS* superfamily (genes encoding Rho proteins) or their regulators (eg, *VAV2* and *VAV3*), were also downregulated.

miRNA Expression Profiling

Within the molecular high-risk group, a unique miRNA expression signature associated with *CEBPA* mutations was derived. It comprised 15 miRNA probes that were upregulated and two that were downregulated in patients with *CEBPA* mutations (false discovery rate = .07; ie, only one of the 17 probes was expected to be falsely positive; Fig 3; Appendix Table A2, online only). Eight of the 15 upregulated miRNA probes corresponded to mature members of the *miR-181* family known to be involved in erythroid and lymphoid lineage differentiation.[39,40] In contrast, no hematopoietic function has been reported for *miR-128*, *miR-192*, *miR219-1-3p*, *miR-224*, *miR-335*, or *miR-340* to date. Of the downregulated miRNAs, *miR-194* has been reported to play a role in intestinal epithelial differentiation, whereas no specific function has been hitherto discovered for *miR-34a*.[41]

DISCUSSION

We report here an independent validation of *CEBPA* gene mutations as favorable outcome predictors in a relatively large group of CN-AML patients treated similarly on CALGB frontline protocols. To our knowledge, this is the first study demonstrating the predictive value of *CEBPA* mutations in CN-AML patients who were also tested for multiple other molecular markers predicting outcome, including *WT1* mutations and changes in *ERG* expression. In previous studies, *CEBPA* mutations provided prognostic information additional to that provided by *FLT3*-ITD and *MLL*-PTD,[22] *FLT3*-ITD and *BAALC* expression levels,[10] and the dual mutational status of *NPM1* mutated/*FLT3*-ITD negative.[15] We showed, using multivariable models, that *CEBPA* mutations independently predict favorable outcome, after adjusting for *FLT3*-ITD, *WT1*, and *NPM1* mutations or *ERG* expression.

Notably, *CEBPA* mutations occur preferentially (> 90%) in the molecular high-risk group of CN-AML patients (ie, patients with *FLT3*-ITD and/or wild-type *NPM1*). In this molecular subset, *CEBPA* mutations provided valuable information in addition to that given by *FLT3*-ITD and *WT1* mutations. Patients with *CEBPA* mutations had significantly lower risk of experiencing induction treatment failure, experiencing relapse, or dying, regardless of the presence or absence of other predictors. These results support risk stratification of CN-AML patients by testing for the presence or absence of *CEBPA*, *NPM1*, and *WT1* mutations and *FLT3*-ITD at diagnosis. CN-AML patients in the molecular low-risk group (ie, with *NPM1* mutations and no *FLT3*-ITD) should also be tested for *ERG* expression because, within this subset, the outcome is excellent for low *ERG* expressers and poor for high *ERG* expressers.[13]

In our analysis, we excluded from the *CEBPA*-mutated group four patients with $CEBPA^{HP196\text{-}197ins}$ as the only *CEBPA* alteration. Recently, $CEBPA^{HP196\text{-}197ins}$ was reported in seven of 19 normal volunteers and in 20 of 100 AML samples;[35] the presence of $CEBPA^{HP196\text{-}197ins}$ was also detected during CR in four patients studied both at diagnosis and during CR. Similarly, $CEBPA^{HP196\text{-}197ins}$ was detected in 22 of 274 nonleukemic blood samples.[36] These findings strongly argue that $CEBPA^{HP196\text{-}197ins}$ represents a polymorphism of the *CEBPA* gene, rather than a bona fide *CEBPA* mutation. Biggio et al[38] showed no significant differences in EFS or OS between CN-AML patients with $CEBPA^{HP196\text{-}197ins}$ and patients with *CEBPA* mutations or with *CEBPA*wt. In our study, there were too few patients to assess clinical outcome of patients with $CEBPA^{HP196\text{-}197ins}$. However, we

Table 3. Multivariable Analyses for Outcome of Cytogenetically Normal Acute Myeloid Leukemia Patients

Variable in Final Models	Hazard Ratio	95% CI	*P*
All patients			
Event-free survival*			
CEBPA, mutated *v* wild type	0.4	0.2 to 0.8	.007
WT1, mutated *v* wild type	2.5	1.4 to 4.7	.003
FLT3-ITD, positive *v* negative	2.1	1.3 to 3.4	.002
ERG expression, high *v* low	1.6	1.0 to 2.6	.04
Age, each 10-year increase	0.6	0.4 to 0.9	.007
Disease-free survival†			
CEBPA, mutated *v* wild type	0.4	0.2 to 0.8	.014
WT1, mutated *v* wild type	3.3	1.7 to 6.6	< .001
FLT3-ITD, positive *v* negative	1.9	1.1 to 3.4	.006
ERG expression, high *v* low	2.7	1.2 to 6.1	.04
Overall survival‡			
CEBPA, mutated *v* wild type	0.3	0.2 to 0.6	< .001
WT1, mutated *v* wild type	3.4	2.3 to 7.0	< .0001
NPM1, mutated *v* wild type	0.4	0.2 to 0.7	.03
FLT3-ITD, positive *v* negative	2.1	1.2 to 3.7	.004
WBC, each 50-unit increase	1.3	1.1 to 1.6	.002
Extramedullary involvement, no *v* yes	2.0	1.2 to 3.2	.01
Molecular high-risk patients			
Event-free survival§			
CEBPA, mutated *v* wild type	0.3	0.2 to 0.6	< .001
WT1, mutated *v* wild type	2.0	1.1 to 3.7	.03
Hemoglobin, each 1-unit increase	0.9	0.9 to 1.0	.04
WBC, each 50-unit increase	1.3	1.0 to 1.6	.03
Disease-free survival‖			
CEBPA, mutated *v* wild type	0.4	0.2 to 0.7	.004
FLT3-ITD, positive *v* negative	1.9	1.0 to 3.6	.008
Hemoglobin	0.8	0.7 to 0.9	.003
Overall survival¶			
CEBPA, mutated *v* wild type	0.4	0.2 to 0.8	.009
WT1, mutated *v* wild type	2.8	1.5 to 5.3	.002
FLT3-ITD, positive *v* negative	2.9	1.3 to 6.6	.009

Hazard ratios greater than 1 indicate higher risk for an event for the first category listed for categorical variables. Hazard ratios less than 1 indicate lower risk for an event for the higher values of a continuous variable. Hazard ratios greater than 1 indicate higher risk for an event for the higher values of a continuous variable. Variables considered in the model were those significant at $\alpha = .20$ from the univariable models. *FLT3*-ITD status and *NPM1* mutation status were evaluated in all final models and included if determined to be confounded with the main analysis variable, *CEBPA*.

Abbreviation: *FLT3*-ITD, internal tandem duplication of the *FLT3* gene.

*Variables considered for model inclusion were *CEBPA* (mutated *v* wild type), *FLT3*-ITD (positive *v* negative), *WT1* (mutated *v* wild type), *ERG* expression (high *v* low), *BAALC* expression (high *v* low), age, hemoglobin, platelets, WBC, and extramedullary involvement based on their significance from univariable analyses. On the basis of clinical importance, *NPM1* (mutated *v* wild type) was tested in the final model and retained because of its confounding effect on *CEBPA*. Age did not meet the proportional hazards assumption and, therefore, was evaluated with an artificial time-dependent covariate in the model. The *P* corresponds to the Wald statistic of a 2-*df* test evaluating whether the coefficients for age and an artificial time-dependent covariate were equal to 0. The hazard ratio presented is for a 10-year increase in age, evaluated at 3 years.

†Variables considered for model inclusion were *CEBPA* (mutated *v* wild type), *FLT3*-ITD (positive *v* negative), *NPM1* (mutated *v* wild type), *WT1* (mutated *v* wild type), *ERG* expression (low *v* high), hemoglobin, WBC, race (white *v* not white), and extramedullary involvement based on their significance from univariable analyses. *NPM1* was retained in the final model along with a time-dependent covariate (because it did not meet the proportional hazards assumption), despite its borderline significance ($P = .067$), because of its confounding effect on *CEBPA*. *FLT3*-ITD did not meet the proportional hazards assumption. The *P* corresponds to the Wald statistic of a 2-*df* test test evaluating whether the coefficients for *FLT3*-ITD and an artificial time-dependent covariate were equal to 0. The hazard ratio presented is for *FLT3*-ITD–positive *v*–negative status, evaluated at 8 months from the date of complete remission. *ERG* did not meet the proportional hazards assumption. The *P* corresponds to the Wald statistic of a 2-*df* test evaluating whether the coefficients for *ERG* and an artificial time-dependent covariate were equal to 0. The hazard ratio provided was evaluated at 1.5 years after achieving complete remission.

‡Variables considered for model inclusion were *CEBPA* (mutated *v* wild type), *FLT3*-ITD (positive *v* negative), *NPM1* (mutated *v* wild type), *WT1* (mutated *v* wild type), *ERG* expression (high *v* low), *BAALC* expression (high *v* low), age, hemoglobin, platelets, WBC, percentage of blood blasts, and extramedullary involvement based on their significance from univariable analyses. *FLT3*-ITD did not meet the proportional hazards assumption and, therefore, was evaluated with an artificial time-dependent covariate in the model. The *P* corresponds to the Wald statistic of a 2-*df* test evaluating whether the coefficients for *FLT3*-ITD and an artificial time-dependent covariate were equal to 0. The hazard ratio presented is for *FLT3*-ITD–positive *v*–negative status, evaluated at 8 months on study. *NPM1* did not meet the proportional hazards assumption and, therefore, was evaluated with an artificial time-dependent covariate in the model. The *P* corresponds to the Wald statistic of a 2-*df* test evaluating whether the coefficients for *NPM1* and an artificial time-dependent covariate were equal to 0. The hazard ratio presented is for *NPM1* mutated *v* wild type, evaluated at 1.5 years on study.

§Variables considered for model inclusion were *CEBPA* (mutated *v* wild type), *FLT3*-ITD (positive *v* negative), *WT1* (mutated *v* wild type), *ERG* expression (high *v* low), hemoglobin, and WBC based on their significance from univariable analyses. On the basis of clinical importance, *NPM1* (mutated *v* wild type) and *FLT3*-ITD (positive *v* negative) were tested in the final model but were not retained because they were not confounded with *CEBPA* and were not significant in the final model.

‖Variables considered for model inclusion were *CEBPA* (mutated *v* wild type), *FLT3*-ITD (positive *v* negative), *FLT3* TKD (positive *v* negative), *WT1* (mutated *v* wild type), hemoglobin, WBC, and race (white *v* not white) based on their significance from univariable analyses. On the basis of clinical importance, *NPM1* (mutated *v* wild type) was tested in the final model but was not retained because it was not confounded with *CEBPA* and was not significant in the final model. *FLT3*-ITD did not meet the proportional hazards assumption and, therefore, was evaluated with an artificial time-dependent covariate in the model. The *P* corresponds to the Wald statistic of a 2-*df* test evaluating whether the coefficients for *FLT3*-ITD and an artificial time-dependent covariate were equal to 0. The hazard ratio presented is for *FLT3*-ITD–positive *v*–negative status, evaluated at 8 months from the date of complete remission.

¶Variables considered for model inclusion were *CEBPA* (mutated *v* wild type), *FLT3*-ITD (positive *v* negative), *WT1* (mutated *v* wild type), *ERG* expression (high *v* low), hemoglobin, platelets, and WBC based on their significance from univariable analyses. On the basis of clinical importance, *NPM1* (mutated *v* wild type) was tested in the final model but was not retained because it was not confounded with *CEBPA* and was not significant in the final model. *FLT3*-ITD did not meet the proportional hazards assumption and, therefore, was evaluated with an artificial time-dependent covariate in the model. The *P* corresponds to the Wald statistic of a 2-*df* test evaluating whether the coefficients for *FLT3*-ITD and an artificial time-dependent covariate were equal to 0. The hazard ratio presented is for *FLT3*-ITD–positive *v*–negative status, evaluated at 9 months on study.

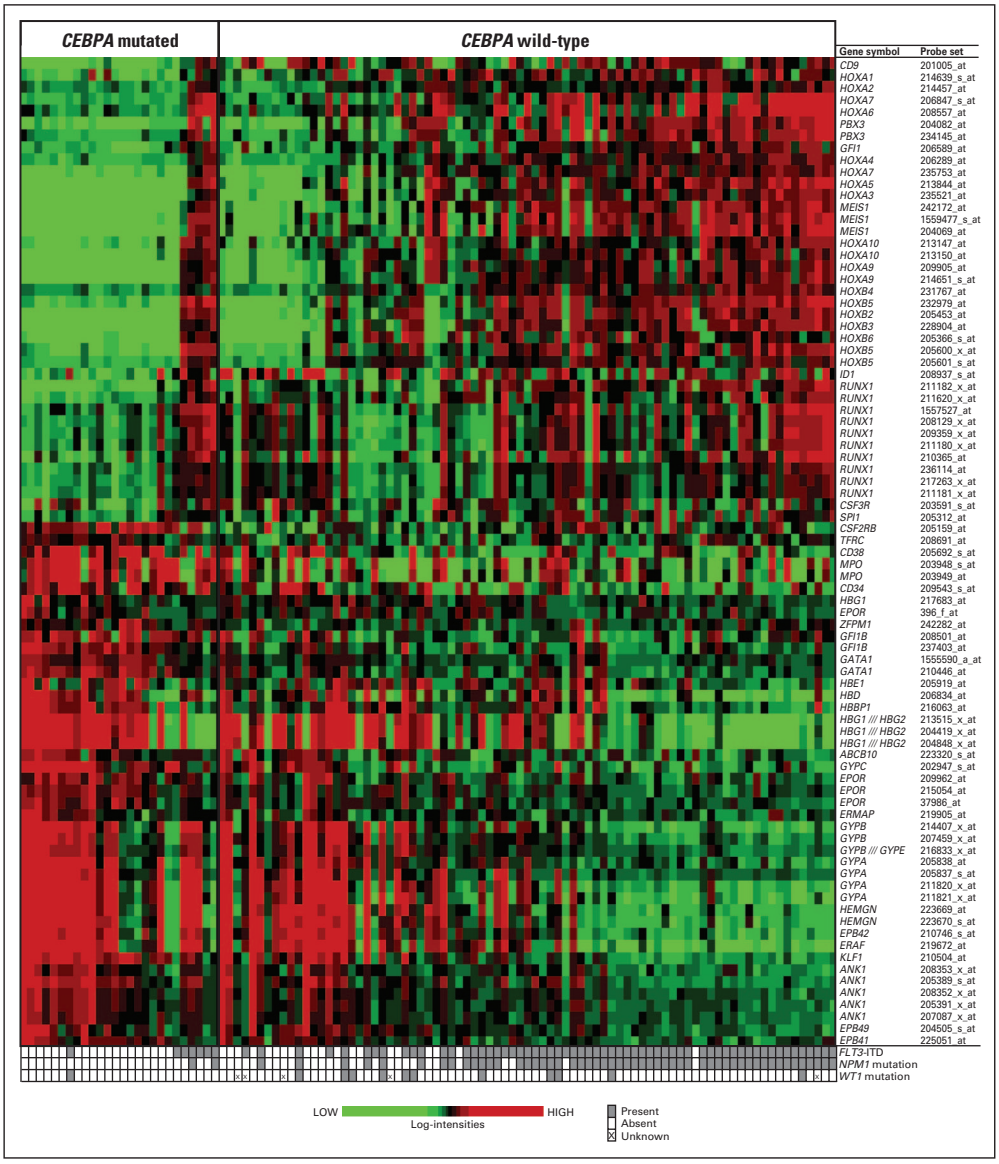

Fig 2. Heat map of selected genes involved in hematopoiesis (ie, erythroid and myeloid lineage and *HOX* genes) from the gene expression signature associated with *CEBPA* mutational status in cytogenetically normal acute myeloid leukemia patients in the molecular high-risk group (ie, patients with *FLT3* internal tandem duplication and/or wild-type *NPM1*). Expression values of the probe sets are represented by color, with green indicating expression less than and red indicating expression greater than the median value for the given probe set. For display purposes, the expression values of the probe sets were centered so that each probe set has the same median expression value. Rows represent probe sets, and columns represent patients. Patients are grouped by *CEBPA* mutational status. Within each *CEBPA* subgroup, patients are ordered according to a summary measure of the expression of the probe sets displayed in the heat map. The summary measure was a linear combination of the expression values of the probe sets, using the base 2 logarithm of the average fold change in expression (*CEBPA* mutations/*CEBPA* wild type) as the coefficient for each expression value.

did not observe significant differences in gene or miRNA expression profiles between patients with $CEBPA^{HP196\text{-}197ins}$ and patients with *CEBPA*wt (data not shown), supporting the notion that $CEBPA^{HP196\text{-}197ins}$ is a normal variant of *CEBPA*wt.

A gene expression signature associated with *CEBPA* mutations was derived in the CN-AML molecular high-risk group. A striking feature of this signature was the previously unreported association of *CEBPA* mutations with the upregulation of several genes involved in erythroid differentiation. This was consistent with the higher hemoglobin levels we observed in patients with *CEBPA* mutations at diagnosis that independently predicted better EFS and DFS in the molecular high-risk group. These results were also consistent with previous laboratory studies investigating hematopoietic differentiation in the absence of *CEBPA* function. Although normal *CEBPA* function is essential for inducing normal myeloid differentiation,[42] hematopoietic cells lacking *CEBPA* function are preferentially redirected toward erythroid differentiation.[43] Wagner et al[44] showed induction of high levels of *GATA1*, which is tightly controlled and upregulated during erythropoiesis,[45,46] and shifting of differentiation from myeloid to erythroid lineage capacity in hematopoietic cells lacking functional *CEBPA*. In accordance with these findings, we report here that, in the CN-AML molecular high-risk group, *CEBPA* mutations were associated with upregulation of *GATA1*, its expression coregulator *ZFPM1*, and other genes involved in erythroid differentiation. *KLF1*, which is required for terminal erythroid differentiation, was upregulated in patients with *CEBPA* mutations along with its target, *EPB49*, and genes encoding the erythropoietin receptor, erythrocyte membrane proteins, hemoglobin chain proteins, and members of the ATP-binding cassette subfamily with a role in heme biosynthesis.[47] In contrast, genes involved in myeloid differentiation, including *RUNX1*, *SPI1*, and *ID1*, a direct target of *CEBPA*,[44] were downregulated. Normal myeloid differentiation requires not only functional *CEBPA*, but also expression of *RUNX1* and *SPI1*.[42] Recent studies reported that *SPI1* is a major downstream target of *RUNX1*[48] and that *GATA1* blocks *SPI1*-mediated gene transactivation.[49] Therefore, it is possible that downregulation of *RUNX1* and *SPI1* and upregulation of

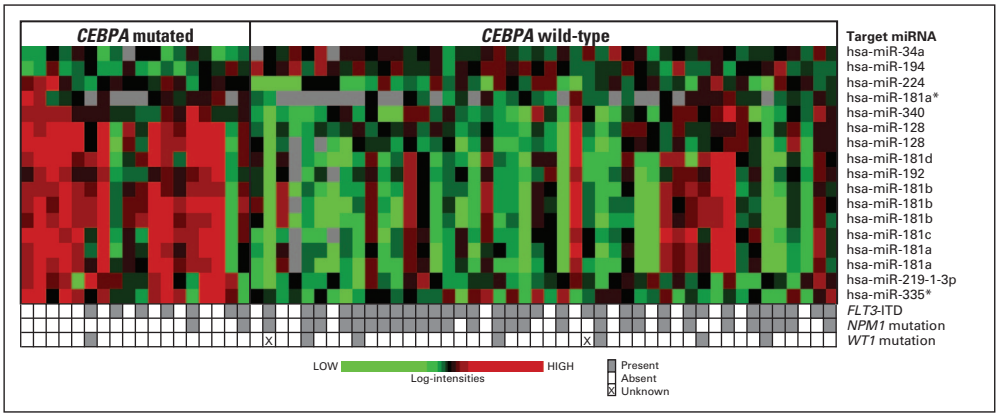

Fig 3. Heat map of the microRNA (miRNA) expression signature associated with *CEBPA* mutational status in cytogenetically normal acute myeloid leukemia patients in the molecular high-risk group (ie, patients with *FLT3* internal tandem duplication and/or wild-type *NPM1*). Expression values of the 17 miRNA probes are represented by color, with green indicating expression less than and red indicating expression greater than the median value for the given probe set. For display purposes, the expression values of the probe sets were centered so that each probe set has the same median expression value. Rows represent probe sets, and columns represent patients. Patients are grouped by the *CEBPA* mutational status.

GATA1 contribute to partial erythroid differentiation of blasts carrying *CEBPA* mutations.

Another prominent feature of the gene expression signature associated with *CEBPA* mutations was the downregulation of several members of the homeobox family, including genes in the *HOXA* and *HOXB* clusters and *MEIS*. These genes have been previously reported to play an important role in the regulation of early stages of hematopoiesis, including the self-renewal of hematopoietic stem cells/early progenitors, and to become silenced at later stages of maturation. These findings, along with upregulation of *CD34* and *CD38*, support our hypothesis that the clonal cell population carrying *CEBPA* mutations is represented by a more mature type of malignant blasts.[50] These findings are also consistent with the significant association of *CEBPA* mutations with *NPM1* wild-type status, which is reportedly accompanied by downregulation of *HOX* gene expression.[51]

We observed that eight of the 17 probes constituting the *CEBPA* mutation–associated miRNA expression signature were members of the *miRNA-181* family. We recently showed that *miR-181a* and *miR-181b* were part of an miRNA expression signature associated with outcome in molecular high-risk CN-AML and that their expression was inversely associated with risk of an event (failure to achieve CR, relapse, or death).[52] However, it is currently unclear whether changes in *miR-181* family expression predict outcome in CN-AML independently of *CEBPA* mutations. Interestingly, Choong et al[40] reported an increase of *miR-181a* and *miR-181b* levels during erythroid differentiation. Therefore, it is reasonable to speculate that overexpression of *miR-181* family contributes to the partial erythroid differentiation of malignant blasts carrying *CEBPA* mutations along with the other aforementioned genes.

In conclusion, the presence of mutated *CEBPA* identifies a subset of patients with better clinical outcome within the molecular high-risk CN-AML group. Consistent with laboratory models, our results of gene expression profiling analysis suggest that lack of functional *CEBPA* as a result of *CEBPA* mutations contributes to partial erythroid differentiation of malignant blasts harboring these mutations. Whether it will be possible to exploit these molecular features to design novel therapeutic approaches specific for patients with *CEBPA* mutations remains to be established. It should also be noted that our results were obtained in younger ($<$ 60 years) patients with primary AML; the frequency and prognostic significance of *CEBPA* mutations in older ($\geq$ 60 years) patients and patients with secondary AML remain to be determined. Nevertheless, we propose that younger CN-AML patients should be routinely screened for *CEBPA* mutations to identify patients who, despite having high-risk molecular features, seem not to require intensive treatments, such as allogeneic stem-cell transplantation, during first CR.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

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

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Appendix

The Appendix is included in the full-text version of this article, available online at www.jco.org. It is not included in the PDF version (via Adobe® Reader®).

