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Brief report

Double *CEBPA* mutations, but not single *CEBPA* mutations, define a subgroup of acute myeloid leukemia with a distinctive gene expression profile that is uniquely associated with a favorable outcome

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Mutations in CCAAT/enhancer binding protein α (*CEBPA*) are seen in 5% to 14% of acute myeloid leukemia (AML) and have been associated with a favorable clinical outcome. Most AMLs with *CEBPA* mutations simultaneously carry 2 mutations (*CEBPA*$^{double\text{-}mut}$), usually biallelic, whereas single heterozygous mutations (*CEBPA*$^{single\text{-}mut}$) are less frequently seen. Using denaturing high-performance liquid chromatography and nucleotide sequencing, we identified among a cohort of 598 newly diagnosed AMLs a subset of 41 *CEBPA* mutant cases (28 *CEBPA*$^{double\text{-}mut}$ and 13 *CEBPA*$^{single\text{-}mut}$ cases). *CEBPA*$^{double\text{-}mut}$ associated with a unique gene expression profile as well as favorable overall and event-free survival, retained in multivariable analysis that included cytogenetic risk, *FLT3*-ITD and *NPM1* mutation, white blood cell count, and age. In contrast, *CEBPA*$^{single\text{-}mut}$ AMLs did not express a discriminating signature and could not be distinguished from wild-type cases as regards clinical outcome. These results demonstrate significant underlying heterogeneity within *CEBPA* mutation-positive AML with prognostic relevance. (Blood. 2009;113:3088-3091)

Introduction

Mutations in the transcription factor CCAAT/enhancer binding protein α (CEBPA) are found in 5% to 14% of acute myeloid leukemia (AML).[1-9] *CEBPA* mutations have been associated with a relatively favorable outcome and have therefore gained interest as a prognostic marker.[4-6,10] Although variable sequence variations have been described, 2 prototypical classes of mutations are most frequent. N-terminal mutations are located between the major translational start codon and a second ATG in the same open reading frame. These mutations introduce a premature stop of translation of the p42 CEBPA protein while preserving translation of a p30 isoform that has been reported to inhibit the function of full-length protein.[2] Mutations in the C-terminal basic leucine zipper (bZIP) region, in contrast, are in-frame and may impair DNA binding and/or homodimerization and heterodimerization.[8] The remaining mutations are mostly found between the N-terminus and the bZIP region.[11]

Most *CEBPA* mutant AMLs exhibit 2 mutations, which most frequently involves a combination of an N-terminal and a bZIP gene mutation.[7,8,11,12] In AMLs with 2 *CEBPA* mutations, the mutations are typically on different alleles.[11] Hence, in these cases, no wild-type CEBPA protein is expressed. A similar condition is found in AMLs carrying a homozygous *CEBPA* mutation.[13] However, there are also AMLs that only show one single heterozygous mutation and thus retain expression of a wild-type allele.[7,11,12]

To obtain better insight into the distribution of the various types of *CEBPA* mutations in de novo adult AML and their impact on clinical outcome, we examined a cohort of 598 cases. After denaturing high-performance liquid chromatography (dHPLC) and nucleotide sequencing, we distinguished cases with 2 different mutations or one homozygous mutation (further referred to as double mutations; *CEBPA*$^{double\text{-}mut}$) as well as cases with only one single heterozygous mutation (*CEBPA*$^{single\text{-}mut}$). Genome-wide gene expression profiling revealed that *CEBPA*$^{double\text{-}mut}$ AMLs expressed a highly characteristic signature, whereas *CEBPA*$^{single\text{-}mut}$ cases did not. In addition, favorable prognosis appeared uniquely associated with *CEBPA*$^{double\text{-}mut}$ AML.

Methods

AML samples, mRNA isolation, dHPLC analysis, and nucleotide sequencing

Bone marrow aspirates or peripheral blood samples of 598 cases of de novo AML were collected, blast cells were purified, and mRNA was isolated as reported.[14] The entire *CEBPA* coding region was investigated by dHPLC and selected regions also by agarose gel analysis and/or nucleotide sequencing. For details on patient characteristics and experimental procedures, see Document S1 (available on the *Blood* website; see the Supplemental Materials link at the top of the online article). All studies were approved by the Erasmus University Medical Center Institutional Review Board (Rotterdam, The Netherlands), and patients' informed consent was obtained in accordance with the Declaration of Helsinki.

Statistical analysis

Survival was estimated according to the method of Kaplan and Meier. The log rank test was used to assess statistical significance. Multivariable analysis was performed using Cox proportional hazards models. Definitions of outcome parameters and cytogenetic risk groups have been described.[15] Further details are given in Document S1. *P* values less than .05 were considered statistically significant.

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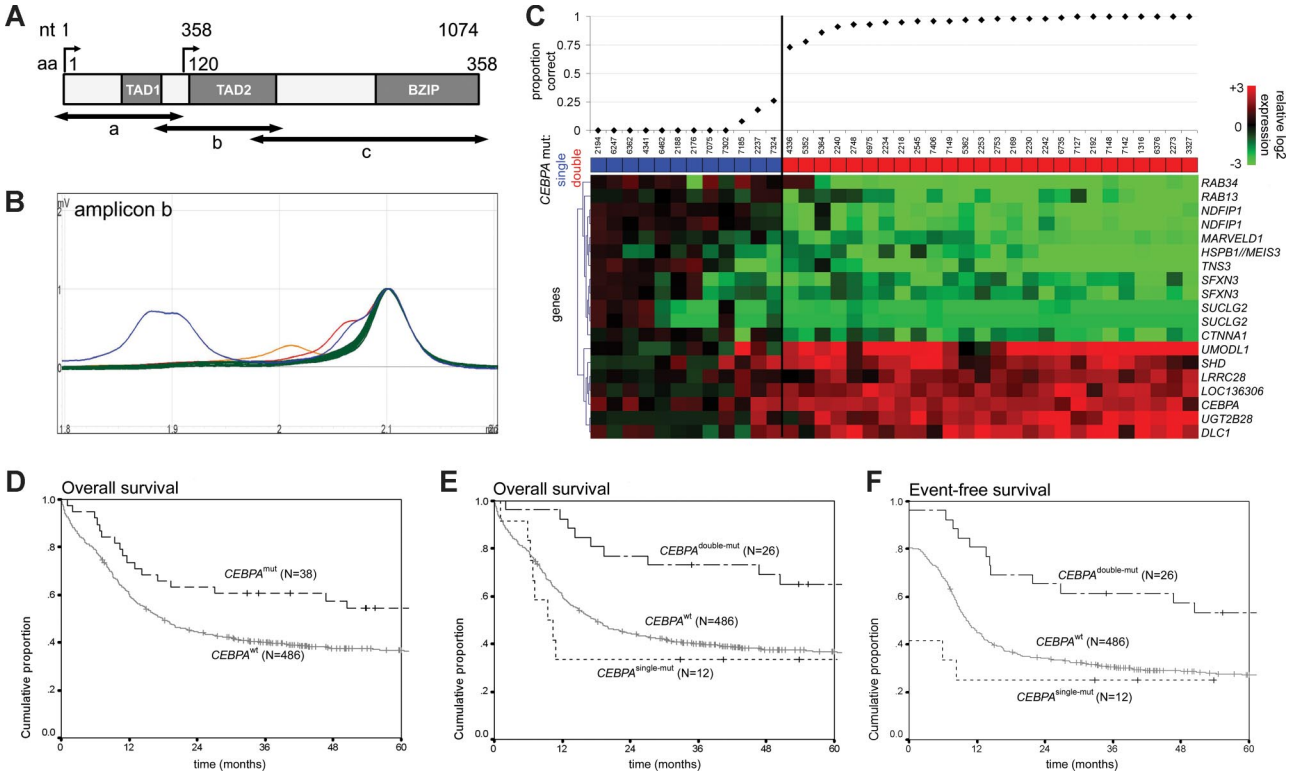

Figure 1. Schematic overview of dHPLC analysis, gene expression profiling analysis, and survival estimates. (A) Schematic representation of the *CEBPA* gene and location of amplicons a, b, and c for polymerase chain reaction, used for dHPLC analysis. Functional regions are depicted: 2 transactivation domains (TAD1 and TAD2) in the N-terminal part, and the bZIP region in the C-terminal part. Nucleotide (nt) position is indicated relative to the main translation start site. Amino acid (aa) numbering and the alternative translation start site at position nt 358 (aa 120) are also depicted. (B) Representative profiles of dHPLC analysis of 1 of the 3 investigated fragments (ie, amplicons b) in a random selection of approximately 90 samples. Heteroduplexes (various colors) are released earlier than homoduplexes (green) and can therefore be recognized as distinct peaks. Time is depicted on the x-axis, and absorbance on the y-axis. (C) A gene expression prediction signature for *CEBPA*^{mut} AML (irrespective of single- or double-mutant status) was derived in a dataset of 524 AMLs, including 38 *CEBPA*^{mut} cases. Prediction accuracy for each of the 38 *CEBPA*^{mut} cases was estimated using repeated 10-fold cross-validation, as detailed in supplemental data. The proportion of correct predictions for the selected 38 *CEBPA*^{mut} specimens is indicated (top panel). Mutation status is color coded (*CEBPA*$^{single\text{-}mut}$, blue; *CEBPA*$^{double\text{-}mut}$, red). The heatmap in the bottom panel depicts the 19 probe sets in the resulting *CEBPA*^{mut} gene expression classifier (Table S2, probe set information). Intensity values (log2) were mean centered over the cohort of 524 AML cases; and for visualization purposes, the genes were hierarchically clustered (Euclidian distance, average linkage). Cells represent relative log 2 expression values and have been color coded on a scale ranging from bright green (−3) to bright red (+3), with black indicating no change relative to the mean. (D) Kaplan-Meier estimates of overall survival among *CEBPA*^{mut} and *CEBPA*^{wt} AML (log rank test, $P = .027$). (E) Overall survival among *CEBPA*$^{double\text{-}mut}$ versus *CEBPA*^{wt} AML ($P = .004$) and versus *CEBPA*$^{single\text{-}mut}$ AML ($P = .005$; pooled $P = .012$). (F) Event-free survival (EFS) among *CEBPA*$^{double\text{-}mut}$ and *CEBPA*^{wt} AML ($P = .005$) and versus *CEBPA*$^{single\text{-}mut}$ AML ($P = .004$; pooled $P = .008$). The cumulative proportion of survival at the intercept (the point where a line crosses the y-axis) reflects the proportion of patients reaching complete remission. Analyses similar to those depicted in panels D-F were performed after splitting the group of *CEBPA*^{wt} AMLs into those with favorable cytogenetics and those with other cytogenetics. These additional analyses can be found in Figure S4.

Gene expression profiling analysis

Gene expression profiles were obtained using Affymetrix (Santa Clara, CA) HGU133Plus2.0 GeneChips. Details on data processing and analysis are given in Document S1.

Results and discussion

In a cohort of 598 cases of adult de novo AML, we identified 65 cases with an aberrant profile in at least 1 of the 3 investigated amplicons of the *CEBPA* coding sequence (Figure 1A,B). The presence of a *CEBPA* sequence variation was confirmed by nucleotide sequencing. Cases that only carried an insertion polymorphism[16,17] or variation(s) that did not lead to amino acid changes were considered wild-type. Two additional specimens were not considered in further analysis because they carried in-frame variations of unknown significance in the N-terminus (Table S1). As a result, 41 of 598 unambiguous *CEBPA*^{mut} AML cases (6.9%) were considered. These included 13 *CEBPA*$^{single\text{-}mut}$ cases and 28 *CEBPA*$^{double\text{-}mut}$ cases. Four of the *CEBPA*$^{double\text{-}mut}$ cases carried homozygous mutations, whereas the remaining 24 cases showed 2 heterozygous mutations (Table S1). Additional screening of the remaining AML cases, using a combination of agarose gel analysis and nucleotide sequencing as described,[6] did not reveal mutations that had been missed by dHPLC.

To investigate whether *CEBPA* mutations related to gene expression, we examined genome-wide gene expression data of 524 AML cases, which included 26 *CEBPA*$^{double\text{-}mut}$ and 12 *CEBPA*$^{single\text{-}mut}$ cases. Clinical and molecular characteristics of the AML cases are reported in Tables S4 and S5. Using prediction analysis for microarrays,[18] according to a supervised approach, we derived a 19-probe set signature predictive of *CEBPA* mutations (Figure 1C). This classifier showed a high specificity (99%) but a limited sensitivity (67%) in cross-validation, indicating a limited ability to recognize all *CEBPA*^{mut} specimens. Strikingly, misclassification was almost entirely the result of *CEBPA*$^{single\text{-}mut}$ cases, whereas *CEBPA*$^{double\text{-}mut}$ AMLs were predicted with an accuracy that was near perfect (Figures 1C, S1). In line with this, we were able to derive a specific 21-probe set classifier for *CEBPA*$^{double\text{-}mut}$ AMLs within the entire AML cohort with a cross-validated sensitivity of 100% (specificity, 98%; Table S3). In further support, unsupervised analysis of the expression data derived from the *CEBPA*^{mut} subset indicated an underlying variability in gene expression that correlated with either double or single mutation status (Figure S2).

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Table 1. Multivariable analysis of *CEBPA*$^{double\text{-}mut}$ and *CEBPA*$^{single\text{-}mut}$ as prognostic markers for overall and event-free survival

	Overall survival		Event-free survival	
Variable	HR (95% CI)	*P*	HR (95% CI)	*P*
CEBPA$^{single\text{-}mut}$*	1.18 (0.58-2.40)	.65	1.61 (0.82-3.17)	.16
CEBPA$^{double\text{-}mut}$*	0.32 (0.17-0.61)	< .001	0.35 (0.20-0.62)	< .001
Intermediate†	2.21 (1.52-3.22)	< .001	2.05 (1.46-2.87)	< .001
Poor†	3.35 (2.27-4.94)	< .001	2.85 (2.00-4.06)	< .001
Age, decades	1.17 (1.08-1.28)	< .001	1.10 (1.02-1.19)	.014
WBC‡	1.33 (1.05-1.68)	.019	1.29 (1.03-1.62)	.025
FLT3 ITD§	1.56 (1.20-2.03)	< .001	1.46 (1.14-1.89)	.003
NPM1 mutation‖	0.55 (0.41-0.74)	< .001	0.51 (0.39-0.67)	< .001

Complete data for multivariable analysis were available for 511 cases.

HR indicates hazard ratio; CI, confidence interval; WBC, white blood cell count; *FLT3*, fms-related tyrosine kinase 3; ITD, internal tandem duplication; and *NPM1*, nucleophosmin.

*_CEBPA_ status versus *CEBPA*^{wt}.

†Cytogenetic risk versus cytogenetic good risk.

‡WBC greater than 20×10^9/L versus less than 20×10^9/L.

§*FLT3* ITD versus no *FLT3* ITD.

‖*NPMI* mutation versus no *NPMI* mutation.

We next assessed how these differences between *CEBPA*$^{double\text{-}mut}$ and *CEBPA*$^{single\text{-}mut}$ related to clinical outcome. In line with previous data, overall survival and event-free survival were significantly better for *CEBPA*^{mut} cases compared with cases with wild-type *CEBPA* (*CEBPA*^{wt}) (Figure 1D; and data not shown). Separate analyses for the *CEBPA*$^{double\text{-}mut}$ and *CEBPA*$^{single\text{-}mut}$ subgroups, however, revealed a favorable outcome that was specific for *CEBPA*$^{double\text{-}mut}$ cases. We failed to find a favorable prognostic effect in relation to the *CEBPA*$^{single\text{-}mut}$ cases. Indeed, *CEBPA*$^{single\text{-}mut}$ AMLs showed a significantly worse outcome than *CEBPA*$^{double\text{-}mut}$ cases, including a poor rate of complete remission (Figure 1E,F). These findings were also apparent in multivariable analysis (Table 1). When only patients younger than 60 years or only patients with normal cytogenetics were considered, similar results were found, although in the latter subgroup with smaller numbers only the pairwise comparison for overall survival between *CEBPA*$^{double\text{-}mut}$ and *CEBPA*$^{single\text{-}mut}$ reached statistical significance (Figure S3; Table S6).

Based on our previous analyses[6] and on the literature,[11] it is probable that, in the majority of the *CEBPA*$^{double\text{-}mut}$ AML studied, both *CEBPA* alleles were affected. A plausible hypothesis is therefore that absence of wild-type *CEBPA* mRNA is directly involved in the *CEBPA*$^{double\text{-}mut}$ gene expression profile. This may be further supported by our previous and current observations that indicate a high degree of similarity between the profiles of *CEBPA*$^{double\text{-}mut}$ AML and a specific subgroup of leukemias characterized by epigenetic *CEBPA* silencing (Figure S1).[19] It is possible that analysis of larger patient series will lead to further refinement of this subclassification, for instance, based on the location of the mutations. For example, our data indicated a tendency of *CEBPA*$^{single\text{-}mut}$ cases with mutations in the bZIP region to be potentially less distinct from the *CEBPA*$^{double\text{-}mut}$ AMLs (case nos. 7185, 7324, and 2237; Figures 1C, S2). Of note, a subset of the *CEBPA*^{mut} AMLs studied here was included in the cohort of 285 cases of AML that we previously investigated by gene expression profiling.[14] In that study, all *CEBPA*$^{double\text{-}mut}$ AMLs were found in 2 particular clusters, whereas *CEBPA*$^{single\text{-}mut}$ AMLs did not specifically aggregate.[14,19]

Studies to date have associated *CEBPA* mutations with outcome[4-6,9] but have not applied subdivisions into single and double mutants. It is unclear why *CEBPA*$^{double\text{-}mut}$ AMLs would have a better outcome than those with a single heterozygous mutation. One explanation could be that a single-mutant *CEBPA* allele is not sufficient for leukemogenesis and requires cooperating mutations, which may be in *CEBPA* itself or in other genes. Of note, recent data indicate that germline *CEBPA* mutations predispose to AML, and the acquisition of a second, somatic *CEBPA* mutation may then contribute to AML development.[20] Indeed, we found a tendency toward more *FLT3*-ITD, *FLT3*-TKD, and *NPM1* mutations in *CEBPA*$^{single\text{-}mut}$ compared with *CEBPA*$^{double\text{-}mut}$ cases (Table S5). Yet unknown abnormalities may associate with *CEBPA*$^{single\text{-}mut}$ AML as well and predispose to inferior outcome. It is however evident that these findings and their clinical significance warrant confirmation in independent cohorts of AML.

In conclusion, the data presented here indicate that *CEBPA*^{mut} AML should at least be distinguished according to the presence of *CEBPA*$^{double\text{-}mut}$ and *CEBPA*$^{single\text{-}mut}$. Screening using dHPLC, followed by nucleotide sequencing, appears useful for rapidly identifying mutant cases. In addition, gene expression-based classification, for instance, using the classifiers described here, enables the accurate identification of *CEBPA*$^{double\text{-}mut}$ AML cases.

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Authorship

Contribution: B.J.W. and C.A.J.E.-V. performed research; B.J.W., C.A.J.E.-V., W.L.J.v.P., P.J.M.V., and R.D. analyzed data; and B.J.W., B.L., P.J.M.V., and R.D. wrote the paper.

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