Characterization of *CEBPA* mutations and promoter hypermethylation in pediatric acute myeloid leukemia

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ABSTRACT

Background

Dysfunctioning of CCAAT/enhancer binding protein α (C/EBPα) in acute myeloid leukemia can be caused, amongst others, by mutations in the encoding gene (*CEBPA*) and by promoter hypermethylation. *CEBPA*-mutated acute myeloid leukemia is associated with a favorable outcome, but this may be restricted to the case of double mutations in *CEBPA* in adult acute myeloid leukemia. In pediatric acute myeloid leukemia, data on the impact of these mutations are limited to one series, and data on promoter hypermethylation are lacking. Our objective was to investigate the characteristics, gene expression profiles and prognostic impact of the different *CEBPA* aberrations in pediatric acute myeloid leukemia.

Design and Methods

We screened a large pediatric cohort (n=252) for *CEBPA* single and double mutations by direct sequencing, and for promoter hypermethylation by methylation-specific polymerase chain reaction. Furthermore, we determined the gene-expression profiles (Affymetrix HGU133 plus 2.0 arrays) of this cohort (n=237).

Results

Thirty-four mutations were identified in 20 out of the 252 cases (7.9%), including 14 double-mutant and 6 single-mutant cases. *CEBPA* double mutations conferred a significantly better 5-year overall survival compared with single mutations (79% *versus* 25%, respectively; *P*=0.04), and compared with *CEBPA* wild-type acute myeloid leukemia excluding core-binding factor cases (47%; *P*=0.07). Multivariate analysis confirmed that the double mutations were an independent favorable prognostic factor for survival (hazard ratio 0.23, *P*=0.04). The combination of screening for promoter hypermethylation and gene expression profiling identified five patients with silenced *CEBPA*, of whom four cases relapsed. All cases characteristically expressed T-lymphoid markers. Moreover, unsupervised clustering of gene expression profiles showed a clustering of *CEBPA* double-mutant and silenced cases, pointing towards a common hallmark of abrogated C/EBPα-functioning in these acute myeloid leukemias.

Conclusions

We showed the independent favorable outcome of patients with *CEBPA* double-mutant acute myeloid leukemia in a large pediatric series. This molecular marker may, therefore, improve risk-group stratification in pediatric acute myeloid leukemia. For the first time, *CEBPA*-silenced cases are suggested to confer a poor outcome in pediatric acute myeloid leukemia, indicating that further investigation of this aberration is needed. Furthermore, clustering of gene expression profiles provided insight into the biological similarities and diversities of the different aberrations in *CEBPA* in pediatric acute myeloid leukemia.

Key words: Pediatric acute myeloid leukemia, *CEBPA* mutation, promoter hypermethylation, molecular marker, prognostic significance.

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Introduction

Current risk-group classification in pediatric acute myeloid leukemia (AML) is determined by recurrent cytogenetic aberrations together with early treatment response.[1] However, the majority of patients are stratified in the intermediate risk group, including patients with cytogenetically normal AML, representing 20-25% of all children with AML. Molecular markers with prognostic implications have been identified in pediatric AML, such as internal tandem duplications of the *FTL3* gene (*FLT3*/ITD), and mutations in *NPM1* and the *Wilms tumor 1* (*WT1*) gene, which may further refine risk-group classification.[2-4]

Mutations in *CEBPA*, encoding the CCAAT/enhancer binding protein alpha (C/EBPα), have also been detected in AML.[5,6] C/EBPα is one of the crucial transcription factors for myeloid cell development. Targeted disruption of the *CEBPA* gene results in a selective early block of granulocyte differentiation.[7,8] C/EBPα function is frequently abrogated in AML by mutations, but also by (post)-transcriptional or post-translational inhibition due to dysregulation by oncogenes such as *AML1-ETO*, *CBF-MYH11* and *FLT3*/ITD.[9-13] More recently, epigenetic modification through hypermethylation of the *CEBPA* promoter, resulting in *CEBPA* silencing, has also been reported.[14,15]

Various mutations throughout *CEBPA* have been described, but two locations are most frequently affected.[16-18] N-terminal frame shift mutations are located between the major translational start site and a second ATG further downstream. They result in truncation of the full-length p42 isoform of C/EBPα, while preserving the shorter p30 isoform, which has been shown to inhibit the function of full-length p42.[9] C-terminal mutations are in-frame insertions or deletions located in the basic leucine zipper (bZIP) domain, and impair DNA binding and/or homo-and heterodimerization.[19] The majority of AML patients with *CEBPA* mutations harbor a mutation at both locations (*CEBPA* double mutants), and these are typically on different alleles, resulting in the lack of wild-type C/EBPα p42 expression in these cases.[20,21] However, single *CEBPA* mutations also occur, in which expression of the wild-type product is retained, albeit at lower levels.

CEBPA mutations are found in 5% to 14% of adult patients with AML, and are associated with a favorable outcome in such patients.[5,6,21-24] In contrast, *CEBPA* promoter hypermethylation has been suggested to confer a poor outcome.[15] Pediatric data are available from two studies, showing *CEBPA* mutations in 4.5% and 6% of cases, and only the Children's Oncology Group reported outcome data according to *CEBPA* status, which confirmed the association with a favorable outcome.[25,26] Recently, two adult studies showed that the favorable prognosis was associated uniquely with *CEBPA* double-mutant AML, but not with the presence of a single *CEBPA* mutation.[27,28] In the Children's Oncology Group study, however, pediatric patients with *CEBPA* single-mutant AML showed a favorable outcome comparable to that of children with double-mutant AML. Pediatric data on *CEBPA* promoter hypermethylation are lacking to date.

Interestingly, adult studies showed a highly characteristic gene expression signature for *CEBPA* double-mutant AML, in contrast to that for single-mutant AML.[27] It is also interesting that *CEBPA* promoter hypermethylated cases showed a similar signature to that for the *CEBPA* double-mutants, which is apparently characterized by the lack of C/EBPα functioning.[15]

In this study we investigated the characteristics, expression profiles and impact of *CEBPA* mutations and promoter hypermethylation in a large series of children with AML.

Design and Methods

Study cohort

Viable frozen bone marrow or peripheral blood samples taken at initial diagnosis from 252 children with AML were provided based on availability by the Dutch Childhood Oncology Group (DCOG; The Hague, the Netherlands), the AML-'Berlin-Frankfurt-Münster' Study Group (AML-BFM-SG; Hannover, Germany, and Prague, Czech Republic) and the Hôpital Saint-Louis (Paris, France). In addition, 33 paired initial diagnosis-relapse bone marrow or peripheral blood samples, and seven paired initial diagnosis-remission bone marrow samples were provided by the DCOG and AML-BFM-SG. Institutional review board approval for these studies was obtained according to local laws and regulations. Each study group performed a central review of the morphological, immunophenotypic and cytogenetic classifications, and provided data on the clinical follow-up.

After thawing, leukemic cells were isolated from these samples as previously described.[29] The percentages of blasts were greater than 80%, as assessed morphologically on May-Grünwald-Giemsa-stained cytospin slides. Genomic DNA and total cellular RNA were extracted using TRIzol reagent (Invitrogen, Breda, the Netherlands), as described before.[30]

Survival analysis was restricted to the patients with *de novo* AML who were treated according to DCOG and AML-BFM-SG studies (i.e. DCOG/AML-BFM 87, DCOG 92/94, DCOG 97, AML-BFM 98 and 04) to reduce treatment variability; these patients accounted for the majority of subjects in our study (n=185). Patients treated according to other protocols (n=43), and, in addition, patients with PML-RARα (n=15) or with secondary AML (n=8) were excluded. Details of the treatment protocols and overall outcome data have already been published, with the exception of those for the AML-BFM 04 study, which was closed recently. In these protocols, treatment consisted of four or five blocks of intensive chemotherapy, using a standard cytarabine and anthracycline backbone. Stem cell transplantation in first complete remission was used only in selected high-risk patients. There was no statistically significant difference between the treatment protocols for obtaining complete remission (P=0.65) or event-free survival (P=0.41), but for overall survival there was a difference between the protocols (P=0.04). However, patients with *CEBPA* single-mutated AML, double-mutated AML and wild-type AML were equally distributed over the different treatment protocols (P=0.28).

Cytogenetic and molecular analysis

Samples were routinely screened for cytogenetic aberrations using standard chromosome-banding karyotyping, and further analyzed for recurrent non-random genetic aberrations characteristic of AML, including t(15;17), inv(16), t(8;21) and *MLL* gene rearrangements, using reverse transcriptase polymerase chain reaction (RT-PCR) and/or fluorescent *in situ* hybridization (FISH), by each study group. In cases of lacking data, RT-PCR or FISH was performed at the laboratory of Pediatric Oncology of the Erasmus MC-Sophia Children's Hospital.

Hotspot regions for mutations of *c-KIT*, *FLT3*, *MLL*, *NPM1*, *PTPN11*, *N-RAS*, *K-RAS* and *WT1* were screened for, as previ-

ously described.[2] Regions of *NOTCH1* known to be mutated in T-cell acute lymphoblastic leukemia [heterodimerization domain (HD), exons 26 and 27; proline-glutamate-serine-threonine-rich domain (PEST), exon 34] were also analyzed for the presence of mutations with a (nested) PCR-based direct sequencing approach.[31]

Analysis of CEBPA mutations and promoter hypermethylation

Mutation analysis of *CEBPA* was performed as previously described,[21] with minor modifications. Primer sequences and PCR conditions are described in *Online Supplementary Table S1*. Genomic DNA was amplified using specific PCR primers, i.e. primers 1 and 10, and 4 and 8, to cover the whole *CEBPA* gene. Purified PCR products were directly sequenced from both strands using the described primers on an ABI Prism 3100 genetic analyzer (Applied Biosystems, Foster City, CA, USA). The sequence data were analyzed using CLC Workbench version 3.5.1 (CLC Bio, Aarhus, Denmark).

For methylation analysis of the promoter region of *CEBPA*, genomic DNA was treated with sodium bisulfite using the EZ DNA Methylation kit (Zymo Research, Orange, CA USA) according to the manufacturer's protocol. The bisulfite-treated DNA was used as a template for methylation-specific PCR and unmethylation-specific PCR, which were performed as previously described.[14] Both methylation-specific and unmethylation-specific PCR products were subsequently separated by gel electrophoresis and visualized with ethidium bromide.

Gene expression profiling and analysis

The integrity of total RNA was checked using the Agilent 2100 Bio-analyzer (Agilent, Santa Clara, CA, USA). Biotinylated cRNA was synthesized, hybridized and processed on the Affymetrix Human Genome U133 Plus 2.0 Array (Affymetrix, Santa Clara, CA, USA) according to the manufacturer's guidelines. Unsupervised clustering analysis was performed and visualized as previously described.[32] Briefly, probe set intensity values were normalized using MAS5.0 software and values less than 30 were set at 30. For each probe set the geometric mean of the intensity values of all samples was calculated. The level of expression of each probe set in every sample was then determined relative to this geometric mean and logarithmically transformed (on a base 2 scale). The transformed expression data were subsequently imported into OmniViz software, (OmniViz v3.7, Tewksbury, MA USA). Pairwise correlations between the gene expression profiles of the 237 samples were calculated with Pearson's correlation on the basis of 1608 differently expressed probe sets representing the subset of probe sets with a 16-factor increase or decrease relative to the geometric mean.

To test the probe set prediction signatures previously described in adult AML for our pediatric *CEBPA* silenced cases and *CEBPA* single- and double-mutant cases, normalized probe set intensities for the 237 cases were used in a linear prediction algorithm (linear discriminant analysis; equal prior probabilities, predicting four variables: Partek v6.09.1008, Missouri, USA), with both the described 21- and 9-probe sets.[15,27] Prediction results of samples were visualized using a principal component analysis scatterplot (Partek), and both cases and probe sets were hierarchically clustered using Euclidean distance (Genemaths XT, Applied Maths, Austin, TX, USA).

Other statistical analyses

Statistical analyses were performed with SPSS 15.0 (SPSS Inc. Chicago, IL, USA). Variables were compared using the χ^2 or Fisher's exact test for categorical values, the Mann-Whitney-U test for continuous values, and the Kruskal-Wallis test when more than two groups were compared.

To assess outcome, the following parameters were used: complete remission (defined as less than 5% blasts in the bone marrow, with regeneration of trilineage hematopoiesis plus absence of leukemic cells in the cerebrospinal fluid or elsewhere), probability of event-free survival (defined as the time between diagnosis and first event, including failure to achieve remission, relapse, death from any cause or second malignancy) and the probability of overall survival (defined as the time between diagnosis and death). The probabilities of event-free and overall survival were estimated by the Kaplan-Meier method, and compared using the log-rank test. The independency of prognostic factors was examined by multivariate Cox regression analysis. All tests were two-tailed and *P* values less than 0.05 were considered statistically significant.

Results

Single and double CEBPA mutations in pediatric acute myeloid leukemia

We identified 34 *CEBPA* mutations in 20 out of 252 (7.9%) diagnostic samples from patients by sequencing the entire coding region (Figure 1A, *Online Supplementary Table S2*). Of these, 13 cases had the combination of an N-terminal frame shift mutation and an in-frame mutation in the bZIP region. One case combined an N-terminal frame shift mutation with a frame shift-causing insertion before the bZIP region. These 14 cases (70%) are henceforth referred to as *CEBPA* double mutants. The other six cases carried a single *CEBPA* mutation: four had in-frame bZIP mutations and two had frame shift mutations in the TAD2 domain and before the bZIP domain, respectively. The latter two cases do not represent the classical N-terminal mutation, as the C/EBPα p30 isoform is also affected, but because of their functional consequence they were classified as mutations.

Cases with an in-frame insertion polymorphism in

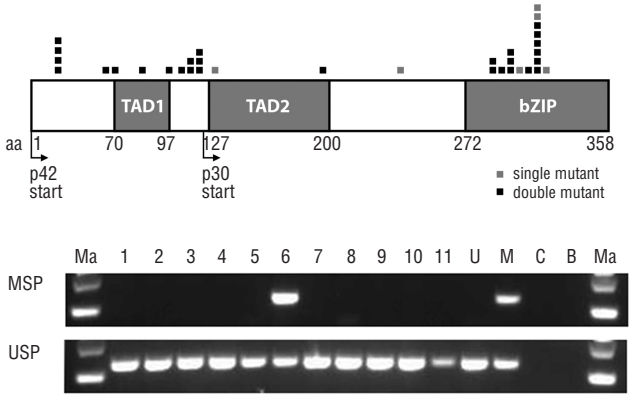

Figure 1. *CEBPA* mutations and promoter hypermethylation in pediatric AML cases. (A) Schematic representation of the *CEBPA* gene and location of the identified mutations. (B) Representative picture of methylation-specific (MSP) and unmethylation-specific PCR (USP) products of the *CEBPA* promoter separated by 2% agarose gel electrophoresis and visualized with ethidium bromide. Patient sample 6 shows a positive MSP product, indicating *CEBPA* promoter hypermethylation. Ma: marker, numbers 1-11: patients' samples, U: unmethylated positive control, M: methylated positive control, C: control bisulfite-untreated DNA, B: blank distilled H_2O.

TAD2[20,33] (n=7), with variation(s) that did not lead to amino acid changes, or with a single amino acid change (situated between the TAD1 and -2 domains) of unknown significance (n=1) were considered to have wild-type *CEBPA*.

Characteristics of CEBPA *single- and double-mutant cases of pediatric acute myeloid leukemia*

The clinical and cell-biological characteristics of the study cohort are shown in Table 1. *CEBPA* double mutations were not present in patients below the age of 3 years. However, the median age of patients with *CEBPA* double-mutant AML (12.3 years) did not differ significantly from that of patients with *CEBPA* single-mutant AML (7.5 years) or with *CEBPA* wild-type AML (9.7 years) (P=0.26). *CEBPA* double mutations occurred exclusively in French-American-British (FAB) types M1 and M2, in contrast to single mutations, which were found in more diverse FAB types (P=0.04). No statistical significant differences were detected between the three subgroups regarding sex and white blood cell count at diagnosis.

Single and double *CEBPA* mutations did not occur in the favorable cytogenetic subgroups [inv(16), t(8;21) and t(15;17)] or in the *MLL*-rearranged subgroup. *CEBPA* double mutations occurred mainly in cytogenetically normal AML (57%), but five cases (36%) also carried an additional cytogenetic aberration (*Online Supplementary Table S2*). In one case (7%) cytogenetic analysis failed, but RT-PCR and/or FISH excluded recurrent cytogenetic aberrations in this case. *CEBPA* single mutations were present in three cases with cytogenetic aberrations (50%), two cases (33%) with a normal karyotype and in one case (17%) cytogenetic analysis failed, but recurrent cytogenetic aberrations were excluded. Additional molecular aberrations were equally frequent in the *CEBPA* single- and double-mutants, and consisted of *FLT3*/ITD, *RAS* and *WT1* mutations, as described in Table 1.

Identification of one case with gain of a single CEBPA *mutation at relapse*

We screened 33 pairs of samples taken at initial diagnosis and relapse; these comprised three *CEBPA*-mutant (2 single and 1 double) and 30 *CEBPA* wild-type cases at initial diagnosis. All three *CEBPA*-mutated cases carried the same mutations at relapse. Of the 30 *CEBPA* wild-type cases at diagnosis, one case (3%) gained an N-terminal frame shift mutation (c.226delG) in *CEBPA* at relapse, i.e. 10 months after diagnosis. The other (cyto)genetic aberrations in this patient (45,X,-X and a *WT1* mutation) were present at both diagnosis and relapse.

Frequency of germ-line origin of CEBPA *mutations*

Of 7 patients with *CEBPA*-mutated AML (4 single and 3 double mutants), remission material (bone marrow mononuclear cells taken in full complete remission) was available. In one case (14%) with *CEBPA* double-mutant AML, the N-terminal frame shift mutation (c.69dupC) was detected in the germ-line material; the second *CEBPA* mutation in this patient, which was located in the bZIP region (c.937_939dupAAG), was somatically acquired in the leukemic cells. An *FLT3*/ITD was also somatically acquired. This patient was diagnosed with AML at the age of 6 years and died 14 months after diagnosis in continuous complete remission because of bleeding. It was not possible to test the *CEBPA* mutational status of the parents as they could not be reached. Interestingly, in unsupervised cluster analysis based on gene expression data (Figure 3), this case (#4746) clustered together with the other *CEBPA* double-mutant cases, indicating that, based on gene expression profiles, the leukemia of this patient was comparable with 'sporadic' *CEBPA* double-mutant AML.

Prognostic impact of CEBPA *single and double mutations in pediatric acute myeloid leukemia*

Survival analysis was restricted to 185 patients with *de novo* AML, including five with *CEBPA* single mutations and ten with *CEBPA* double mutations (*Online Supplementary Tables S2 and S3*). The median follow-up period of the survivors was 4.4 years. All ten patients with *CEBPA* double mutations reached complete remission (100%), while complete remission was achieved in four

Table 1. Characteristics of the 252 pediatric AML patients included in this study, divided by *CEBPA* mutation status.

	All	*CEBPA* single mutation	*CEBPA* double mutation	*CEBPA* wild-type	*P* value
Number	252	6	14	232	
Age, median (years)	9.7	7.5	12.3	9.7	0.26*
<3 years, n (%)	49	1 (17%)	-	48 (21%)	
≥3 years, n (%)	203	5 (83%)	14 (100%)	184 (79%)	0.16#
Sex (% female)	45.2%	66.7%	42.9%	44.8%	0.56#
WBC (x10^9/L), median (range)	42 (0-535)	20 (8-535)	60 (6-388)	41 (0-483)	0.69*
FAB classification, n(%)					0.001#
M0	12 (5%)	1 (17%)	-	11 (5%)	
M1	27 (11%)	2 (33%)	6 (43%)	19 (9%)	
M2	55 (23%)	1 (17%)	8 (57%)	46 (21%)	
M3	20 (8%)	-	-	20 (9%)	
M4	60 (25%)	2 (33%)	-	58 (26%)	
M5	57 (24%)	-	-	57 (26%)	
M6	3 (1%)	-	-	3 (1%)	
M7	8 (3%)	-	-	8 (4%)	
other	1 (0%)		-	1 (0%)	
Unknown	9 (4%)	-	-	9 (4%)	
Karyotype, n(%)					0.049#
t(8;21)	27 (11%)	-	-	27 (12%)	
inv(16)	26 (10%)	-	-	26 (11%)	
t(15;17)	18 (7%)	-	-	18 (8%)	
11q23	49 (19%)	-	-	49 (21%)	
normal	55 (22%)	2 (33%)	8 (57%)	45 (19%)	
other	60 (24%)	3 (50%)	5 (36%)	49 (21%)	
unknown	17 (7%)	1 (17%)	1 (7%)	15 (7%)	
FLT3/ITD, n(%) (n=252)	52 (21%)	1 (17%)	3 (21%)	48 (21%)	0.97#
N- or *K-RAS*, n(%) (n=251)	52 (21%)	1 (17%)	2 (14%)	49 (21%)	0.80#
c-KIT , n(%) (n=251)	17 (7%)	-	-	17 (7%)	0.45#
MLL-PTD, n(%) (n=244)	5 (2%)	-	-	5 (2%)	0.80#
NPM1, n(%) (n=247)	18 (7%)	-	-	18 (8%)	0.43#
WT1, n(%) (n=250)	27 (11%)	2(33%)	3 (21%)	22 (10%)	0.08#

**Kruskal-Wallis test; #Chi-square test; WBC: white blood cell count at diagnosis; FAB: French-American British.*

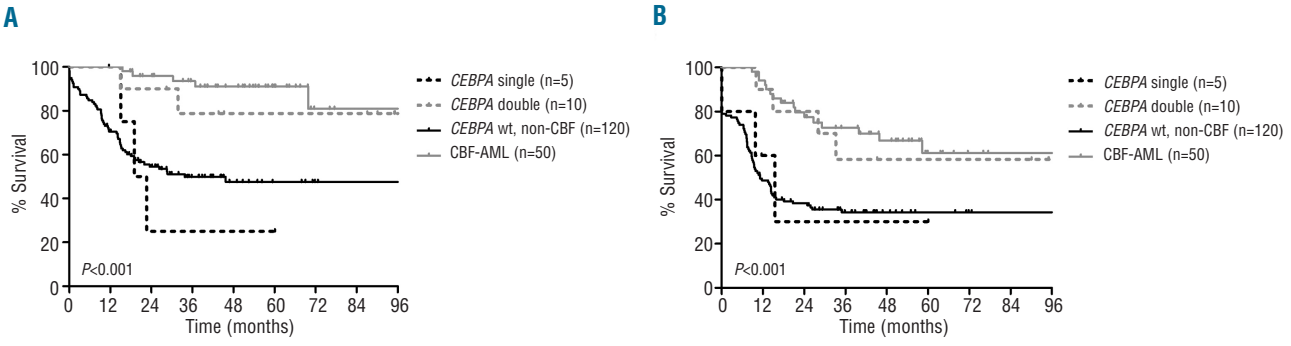

Figure 2. Kaplan-Meier survival curves of overall survival and event-free survival according to *CEBPA* status in pediatric AML. Probability of (A) overall survival and (B) event-free survival of four subgroups of patients with pediatric AML, i.e. *CEBPA* single-mutant AML, *CEBPA* double-mutant AML, *CEBPA* wild-type non-core-binding factor (CBF) AML and CBF-AML.

out of the five (80%) patients with *CEBPA* single mutations, as one patient had resistant disease. The complete remission rate for *CEBPA* wild-type patients (n=170) was 84%.

Patients with *CEBPA* double-mutant AML had a significantly better 5-year overall survival compared with those with a single *CEBPA* mutation (5-year probability of overall survival 79±13% *versus* 25±22%; *P*=0.04), although the 5-year event-free survival was not significantly different (5-year probability of event-free survival 58±16% *versus* 30%±24%; *P*=0.16) (Figure 2). In fact, the outcome of patients with *CEBPA* double-mutations was comparable to that of patients in the favorable-risk group with core-binding factor AML [inv(16) or t(8;21)], who had a 5-year probability of overall survival of 91±4%; *P*=0.51 and of event-free survival of 61±9%; *P*=0.74. Furthermore, patients with *CEBPA* double mutations showed a clear trend to a more favorable outcome than patients with wild-type *CEBPA* after excluding the core-binding factor-AML cases (5-year probability of overall survival 47±5%; *P*=0.07 and of event-free survival 33±4%; *P*=0.06). The impact of additional molecular or cytogenetic aberrations (e.g. *FLT3*/ITD) on the *CEBPA* single- and double-mutated group could not be investigated due to small numbers.

Multivariate analysis, including age, white blood cell count at diagnosis, favorable cytogenetics, *NPM1* mutations and *FLT3*/ITD, showed that the presence of a *CEBPA* double mutation was an independent favorable prognostic factor for overall survival (HR 0.23; *P*=0.04) as well as event-free survival (HR 0.32; *P*=0.03) (Table 2). *CEBPA* single mutations were not included in the multivariate analysis as a factor because of the small number of cases.

Aberrant CEBPA *promoter hypermethylation in pediatric acute myeloid leukemia*

Methylation-specific PCR could be performed in 237 cases and revealed hypermethylation of the *CEBPA* promoter region in only three cases (1.3%) (Figure 1B). As expected, *CEBPA* gene expression (determined with probe set 204039_at and depicted in Figure 3) was down-regulated in these cases. The characteristics of these three *CEBPA*-hypermethylated cases are shown in Table 3. *CEBPA* promoter hypermethylation was also present in the relapse material (n=2) from these patients, demonstrating clonal stability of the hypermethylation pattern.

Table 2. Results of multivariate analysis for overall survival (OS) and event-free survival (EFS).

Outcome	Variable	Hazard ratio	95% confidence interval	*P* value
OS	Favorable karyotype	0.11	0.04-0.30	<0.001
	CEBPA double mutation	0.23	0.06-0.96	0.04
	NPM1 mutation	0.43	0.17-1.09	0.08
	WBC >50×10⁹/L	1.23	0.76-2.10	0.36
	FLT3/ITD	1.28	0.70-2.34	0.42
	Age >10 years	1.07	0.64-1.79	0.79
EFS	Favorable karyotype	0.27	0-15-0.47	<0.001
	NPM1 mutation	0.29	0.13-0.69	0.005
	CEBPA double mutation	0.32	0.12-0.89	0.03
	FLT3/ITD	1.34	0.81-2.23	0.25
	Age >10 years	1.18	0.78-1.78	0.46
	WBC >50×10⁹/L	1.03	0.68-1.54	0.90

WBC: white blood cell count; OS: overall survival; EFS: event-free survival.

Unsupervised analysis reveals clustering of CEBPA *mutant and hypermethylated cases*

Unsupervised cluster analysis of 237 children with *de novo* AML showed distinct clusters (Figure 3A). Cases with *CEBPA* mutations and promoter hypermethylation predominantly clustered together, and are referred to as the main *CEBPA* cluster. This cluster contained 15 cases in total, including eight double-mutants and all three hypermethylated cases. Of interest, two cases of *CEBPA* single-mutant AML were also present in this main *CEBPA* cluster, despite the fact that these cases are expected to have wild-type expression of C/EBPα p42, in contrast to *CEBPA* double-mutant and hypermethylated cases. Interestingly, extremely high *TRIB2* expression (probe set 202478_at) was present in one of these *CEBPA* single-mutant cases (#5041), which may explain the C/EBPα p42-inhibition of its remaining allele, as *TRIB2* directly inactivates C/EBPα p42.[34]

Furthermore, the two remaining cases in the *CEBPA* main cluster had low *CEBPA* gene expression, and clustered closely with the three cases of *CEBPA*-hypermethylated AML, despite the fact that we did not detect hypermethylation in these cases using methylation-specific PCR (Figure 3B, Table 3). Of note, one of these cases also had very high *TRIB2* expression (#4728). So, taken togeth-

er, five patients with silenced *CEBPA* were found among 237 cases of *de novo* pediatric AML (2.1%). Four of these patients experienced a relapse, and only one patient was in continuous complete remission after hematopoietic stem cell transplantation (Table 3).

Clearly, a common gene expression signature was shared for all the cases in the *CEBPA* main cluster, which was confirmed when comparing this cluster with all others (*Online Supplementary Figure S1, Online Supplementary Table S4*).

T-cell characteristics of CEBPA-silenced cases in pediatric acute myeloid leukemia

As *CEBPA*-silenced cases were reported to express T-cell lineage genes and *NOTCH1* mutations in adults, we next investigated T-cell characteristics and screened for *NOTCH1* mutations in our five pediatric cases with silenced *CEBPA*.[15] Flow cytometry data revealed high CD7 expression in all five cases besides myeloid (CD33/CD13 and CD11b) and stem-cell markers (CD34 and CD117) (*Online Supplementary Table S5*). One case expressed cCD3 weakly (#5033), but expression of other T-cell antigens was not seen. However, high mRNA expression of *CD3* (*CD3Z*: 210031_at, *CD3G*: 206804_at and *CD3D*: 213539_at) was seen in all cases (*Online Supplementary Table S5*). High *LCK* expression, which is a well-known T-lineage marker (probe set 204891_s_at),[35] was also found in all five cases. We did not detect *NOTCH1* mutations in the HD or PEST domain in our five *CEBPA*-silenced cases.

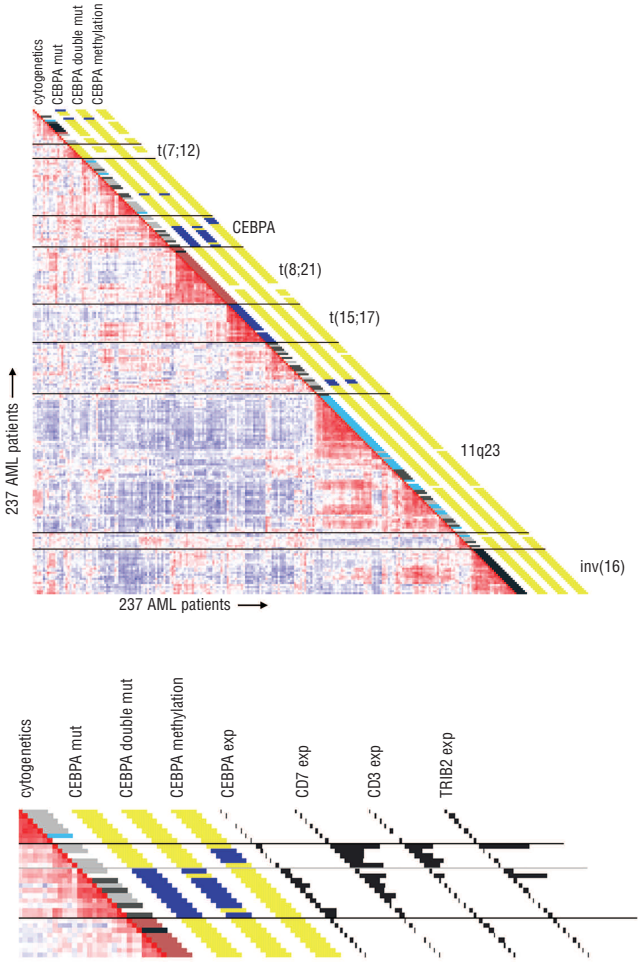

Figure 3. Unsupervised clustering of gene expression data revealed clustering of cases with aberrant *CEBPA* predominantly in one cluster in pediatric *de novo* AML. (A) Pair wise correlations between gene expression profiles of 237 *de novo* pediatric AML samples, calculated on the basis of 1608 probe sets (cutoff: 16-fold), are displayed in a correlation plot. Colors of boxes represent the Pearson's correlation coefficient with a color gradient ranging from deep blue for a negative correlation, to vivid red for a positive correlation. Distinct clusters of samples, which can be recognized by the red blocks showing strong correlation along the diagonal, are observed. The first column to the right of the plot indicates the major cytogenetic subgroup the samples belong to [dark blue: inv(16), pale red: t(8;21), bright blue t(15;17), yellow: t(7;12), light blue: 11q23, dark gray: normal cytogenetics, light gray: other cytogenetic aberrations and white: failure]. Clustering of these cytogenetic subgroups is seen. The second, third and fourth columns represent presence (blue) or absence (yellow) of a *CEBPA* mutation, *CEBPA* double mutation and *CEBPA* promoter methylation, respectively. The majority of *CEBPA* mutant cases aggregated together with all *CEBPA* hypermethylated cases in one main cluster. (B) An enlarged view of the correlation plot focusing on the *CEBPA* main cluster is shown. The order of the patients' samples in this cluster from top to bottom is as follows: #4728, #5033, #3496, #3451, #4736, #3439, #5041, #5061, #4746, #5063, #5047, #4396, #4445, #4747, #5013. Additionally, four histograms show the expression of *CEBPA* (204039_at), *CD7* (214551_s_at), *CD3D* (213539_at) and *TRIB2* (202478_at). Within the *CEBPA* main cluster, two sub-clusters based on expression of *CEBPA* can be identified and are separated by a gray line, i.e. *CEBPA* low or absent expression (n=5), including the three *CEBPA* promoter methylated cases, and *CEBPA* high expression with the *CEBPA* mutant cases (n=10). The five silenced *CEBPA* cases are further characterized by high *CD3* and *CD7* expression. Silenced case #4728 and single-mutated case #5041 show high *TRIB2* expression, which has been shown to inhibit C/EBPα p42.

Table 3. Characteristics of the five *CEBPA*-silenced AML cases, including three cases with *CEBPA* promoter hypermethylation.

ID	MSP*	Age (years)	Sex	WBC (x10⁹/L)	FAB	Karyotype	Molecular aberration	Treatment protocol	Follow-up
#3451	+	6,3	male	196	M0	46,XY,del(17)(p12p13) or add(17)(p11)[2]	WT1, N-RAS	DCOG97	relapse after 11.7 months
#3496	+	11,3	female	NA	M5	NA	WT1, K-RAS	DCOG97	relapse after 8.2 months
#5033	+	10,3	female	NA	M0	46,XX,t(3;4)(q11-12;p15-16)[6]/ 47,XX,t(3;4)(q11-12;p15-16),+mar[12]/ 46,XX[2]	none	LAME	CCR for 8.5 years (stem cell transplant 8 months after diagnosis)
#4728	-	9,7	male	2.4	M0	47,XY,inv(12)(p1?3,q1?3),+19[6]/46,XY[14]	none	DCOG97	relapse after 38.4 months
#4736	-	8,5	female	2.9	M4	46,XX,del(7)?(p13;p21)[1], idem + t(3;7)? (p25;p15)[11], idem + del(11)?(p11p14)[13]	none	DCOG97	relapse after 7.3 months

**MSP: methylation-specific PCR for* CEBPA *promoter hypermethylation: + positive, - negative; NA: not available; WBC: white blood cell count; CCR: continuos complete remission.*

Prediction of CEBPA *double-mutant and -silenced cases using adult acute myeloid leukemia gene signatures*

We utilized previously established gene prediction signatures in adult AML, based on 21- and 9-probe sets for *CEBPA* double-mutant and *CEBPA*-silenced cases, respectively.[15,27] Of the 12 *CEBPA* double-mutant cases, ten were correctly predicted using the 21-probe set-containing signature, one was predicted as being a single mutant, and one as a silenced case (sensitivity 83%, specificity 99%) (*Online Supplementary Table S6A*). Visualizing these results, it can be seen that the double-mutant cases form a main cluster apart from the wild-type cases (*Online Supplementary Figure S2A,B*). However, three *CEBPA* single-mutant cases (1 predicted as a double mutant), with the single mutation located in the bZIP region, also clustered with the *CEBPA* double-mutant cases.

The 9-probe set-signature for *CEBPA*-silenced cases predicted three of our five silenced cases, but also one *CEBPA* wild-type was false positively recognized (*Online Supplementary Table S6B, Online Supplementary Figure S2C,D*). This resulted in a low sensitivity (60%) of these probe sets for the prediction of *CEBPA*-silenced cases in our pediatric series.

Discussion

In this study we investigated *CEBPA* aberrations in pediatric AML to determine their frequency and prognostic impact, and also to gain further insight into the biology of pediatric AML with *CEBPA* aberrations. We detected *CEBPA* mutations in 7.9% of pediatric AML cases, which is comparable to the reported frequency in adult AML (5-14%),[9,21-24] and the two available pediatric series from Taiwan (6%) and North-America (4.5%).[25,26] Seventy percent of *CEBPA*-mutated cases carried a double mutation, which is in agreement with previous studies reporting that the majority of *CEBPA*-mutated cases carried double mutations, typically affecting both alleles.[20,21]

Recently, two reports on adult series postulated that *CEBPA* single- and double-mutant AML are different entities, as a favorable outcome was associated uniquely with *CEBPA* double mutants.[27,28] Moreover, patients with double mutations were characterized by a specific gene expression signature, in contrast to those with *CEBPA* single mutations. This is further sustained by recently published data from mouse models that showed an efficient synergistic effect of the two different *CEBPA* mutations on leukemic transformation.[36-38] Single *CEBPA* mutations are, however, believed to predispose the pre-leukemic initiating cell to subsequent acquirement of secondary (epi)genetic mutations necessary before the development of full-blown AML.[37-38] Despite small numbers, we observed differences, both in presenting characteristics as well as in prognosis, between *CEBPA* single- and double-mutant AML. With regards to presenting characteristics, *CEBPA* double-mutant AML did not occur in very young patients, was restricted to FAB M1/M2 subtypes, and had the strongest association with cytogenetically normal AML. However, the frequency of additional molecular mutations was not higher in *CEBPA* single-mutant cases than in *CEBPA* double-mutant cases.

With regards to prognosis, only patients with *CEBPA* double mutations were associated with a favorable outcome, with a 5-year overall survival of 79%. Those with single mutations had a relatively poor outcome in our series (5-year probability of overall survival of 25%). In fact, the outcome of the *CEBPA* double-mutant cases was comparable to that of the subgroup with the favorable core-binding factor-AML. This is in agreement with the results of the pediatric Children's Oncology Group study,[25] which showed an overall survival of approximately 80% at 5 years for patients with *CEBPA* double-mutant AML. Multivariate analysis confirmed the independent prognostic significance of *CEBPA* double mutations, which points to its potential as a marker for further refinement of risk-group stratification in pediatric AML, when validated in prospective series.

The mechanism of the relative drug sensitivity of the *CEBPA* double-mutated cases remains to be elucidated. The outcome of cases with a single mutation in the series reported by the Children's Oncology Group (5-year predicted overall survival of 85%) was similar to that of the cases with double mutations. Despite the fact that the numbers of single-mutant cases in both series were small, we hypothesize that the difference in outcome between our studies may be based on the underlying biology of the type of the single mutation (i.e. a mutation at the N-terminus, bZIP region or other location) as different leukemogenic capacities have been associated with the different types of mutation,[37] or by different cooperating genetic events. Further studies of the single-mutant group in pediatric and adult AML are clearly warranted to determine the impact of the different mutation types and cooperating genetic aberrations.

Germ-line *CEBPA* mutations have been discovered in familial AML, in which the N-terminal mutation is present in the germ-line, and frequently a second *CEBPA* bZIP mutation is somatically acquired as a second hit to develop AML.[39,40] The frequency of germ-line mutations in adult AML with *CEBPA* mutations was estimated at 11% (2 cases out of 18).[41] Here we found a similar frequency: one out of seven cases of pediatric *CEBPA*-mutated AML had a germ-line mutation. In our pediatric case we also found an N-terminal mutation in the germ-line, and a somatically acquired bZIP mutation. The occurrence of AML in both children and adults with germ-line *CEBPA* mutations illustrates a variable latency time.

Promoter hypermethylation of *CEBPA* was present at a low frequency in our pediatric series. These hypermethylated cases showed *CEBPA*-silencing and, utilizing unsupervised clustering of gene expression data, clustered together with the double-mutant cases. Two other cases with *CEBPA*-silencing without promoter hypermethylation were detected; these two cases clustered together with the hypermethylated cases. The mechanism of gene silencing in these two cases still has to be elucidated, but could be hypermethylation in other regions of the promoter,[42] silencing by other epigenetic processes or by binding of microRNA. All five *CEBPA*-silenced cases had T-lymphoid characteristics beside their myeloid and stem cell markers. However, no *NOTCH1* mutations were detected, which may be due to the small number of cases as *NOTCH1* mutations were present in only 50% of adult cases with silenced *CEBPA*.[15] In contrast to the favorable outcome of patients with *CEBPA* double mutations, four out of the five *CEBPA*-silenced cases experienced a relapse. Interestingly, the patient who did not relapse had received a stem cell transplant. Cases with silenced *CEBPA* due to hypermethylation might potentially benefit from the use of demethylating agents.

Clustering of *CEBPA*-silenced and double-mutant cases points towards a common feature of C/EBPα inactivation in these leukemias. Cases in this *CEBPA* main cluster clearly shared a specific gene expression profile. However, the main cluster could also be divided into two sub-clusters, separating the double-mutant and silenced cases, which might also underlie biological factors influencing drug resistance and thereby the difference in prognosis between the two subgroups. Differences in methylation profiles have already been shown between these two subgroups.[43] Interestingly, two *CEBPA* single-mutant cases, which are expected to have full-length C/EBPα p42 expression of the unaffected allele, also aggregated in this cluster. In one of these cases, high *TRIB2* expression was detected, which is known to directly inactivate the C/EBPα p42 isoform.[34] Complete C/EBPα p42 inactivation of the wild-type allele is hereby established and clustering with cases of AML without functional C/EBPα p42 can be explained. The mechanism of the expected C/EBPα p42 inactivation in the other single-mutant case remains to be elucidated.

We tried to predict *CEBPA* double-mutant and silenced cases in our pediatric series based on a prediction signature derived from adult studies. A high sensitivity and specificity was reached for *CEBPA* double-mutants, although one single-mutant case was also falsely predicted, and two single-mutant cases clustered with the double-mutant cases. These three cases did, however, carry a mutation in the bZIP region, which was previously shown to have a tendency towards a *CEBPA* double-mutant gene expression profile.[27] Prediction of the *CEBPA*-silenced cases was difficult due to a low sensitivity.

In conclusion, we showed the independent favorable outcome of patients with *CEBPA* double-mutant AML in a large series of pediatric AML. Hence, *CEBPA* double mutations may improve risk-group stratification in pediatric AML, if these data are validated in prospective series. For the first time, *CEBPA*-silencing is suggested to confer a poor outcome in pediatric AML, warranting further investigation of this *CEBPA* aberration. Furthermore, clustering of gene expression profiles provided insight into the biological similarities and diversities of *CEBPA* aberrations in pediatric AML.

Authorship and Disclosures

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