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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=257).

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. 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 FLT3 gene (FLT3/ITD), and mutations in NPM1 and the Wilms tumor 1 (WT1) gene, which may further refine risk-group classification.24

Mutations in \(\text{CEBPA}\), encoding the CCAAT/enhancer binding protein alpha (C/EBP\(\alpha\)), have also been detected in AML.\(^2\) C/EBP\(\alpha\) is one of the crucial transcription factors for myeloid cell development. Targeted disruption of the \(\text{CEBPA}\) gene results in a selective early block of granulocyte differentiation.\(^7\) C/EBP\(\alpha\) function is frequently abrogated in AML by mutations, but also by (post)-transcriptional or post-translational inhibition due to dysregulation by oncogenes such as \(\text{AML}_{1}-\text{ETO}, \text{CBF}-\text{MYH11}\) and \(\text{FLT3}/\text{ITD}\).\(^25\) More recently, epigenetic modification through hypermethylation of the \(\text{CEBPA}\) promoter, resulting in \(\text{CEBPA}\) silencing, has also been reported.\(^14\)\(^15\)

Various mutations throughout \(\text{CEBPA}\) have been described, but two locations are most frequently affected.\(^16\)\(^11\) 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/EBP\(\alpha\), while preserving the shorter p30 isoform, which has been shown to inhibit the function of full-length p42.\(^6\) 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 \(\text{CEBPA}\) mutations harbor a mutation at both locations (\(\text{CEBPA}\) double mutants), and these are typically on different alleles, resulting in the lack of wild-type C/EBP\(\alpha\) p42 expression in these cases.\(^20\)\(^21\) However, single \(\text{CEBPA}\) mutations also occur, in which expression of the wild-type product is retained, albeit at lower levels. \(\text{CEBPA}\) mutations are found in 5% to 14% of adult patients with AML, and are associated with a favorable outcome in such patients.\(^5\)\(^6\)\(^6\)\(^24\) In contrast, \(\text{CEBPA}\) promoter hypermethylation has been suggested to confer a poor outcome.\(^22\)\(^23\) Pediatric data are available from two studies, showing \(\text{CEBPA}\) mutations in 4.5% and 6% of cases, and only the Children’s Oncology Group reported outcome data according to \(\text{CEBPA}\) status, which confirmed the association with a favorable outcome.\(^25\)\(^26\) Recently, two adult studies showed that the favorable prognosis was associated uniquely with \(\text{CEBPA}\) double-mutant AML, but not with the presence of a single \(\text{CEBPA}\) mutation.\(^27\)\(^28\) In the Children’s Oncology Group study, however, pediatric patients with \(\text{CEBPA}\) single-mutant AML showed a favorable outcome comparable to that of children with double-mutant AML. Pediatric data on \(\text{CEBPA}\) promoter hypermethylation are lacking to date.

Interestingly, adult studies showed a highly characteristic gene expression signature for \(\text{CEBPA}\) double-mutant AML, in contrast to that for single-mutant AML.\(^23\) It is also interesting that \(\text{CEBPA}\) promoter hypermethylated cases showed a similar signature to that for the \(\text{CEBPA}\) double-mutants, which is apparently characterized by the lack of C/EBP\(\alpha\) functioning.\(^3\)

In this study we investigated the characteristics, expression profiles and impact of \(\text{CEBPA}\) 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-BFM-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 cytosin 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 \textit{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-RAR\(\alpha\) (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 \(\text{CEBPA}\) 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 \(\text{MLL}\) gene rearrangements, using reverse transcriptase polymerase chain reaction (RT-PCR) and/or fluorescent \textit{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, \(\text{MLL}\), NPM1, \(\text{PTPN}11\), \(\text{N-RAS}\), \(\text{K-RAS}\) and WT1 were screened for, as previ-
ously described. 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 (PETS), exon 34] were also analyzed for
the presence of mutations with a (nested) PCR-based direct
sequencing approach.

Analysis of CEBPA mutations and promoter hypermethylation

Mutation analysis of CEBPA was performed as previously
described, 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 5.5.1 (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) accord-
ing to the manufacturer’s protocol. The bisulfite-treated DNA
was used as a template for methylation-specific PCR and unmethyla-
tion-specific PCR, which were performed as previously described.
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. Briefly, probe set intensity values were nor-
malized using MAS5.0 software and values less than 30 were set
at 30. For each probe set the geometric mean of the intensity val-
ues 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 OmnimViz software, (OmnimViz 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 10-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. Prediction results of samples
were visualized using a principal component analysis scatterplot
(Partek), and both cases and probe sets were hierarchically clus-
tered 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 χ² 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 over-
all 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 multi-
-variate 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 lat-
ter 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 classi-

Figure 1. CEBPA mutations and promoter hypermethylation in pedi-
iatric AML cases. (A) Schematic representation of the CEBPA gene
and location of the identified mutations. (B) Representative picture of
methylationspecific (MSP) and unmethylationspecific PCR (USP)
products of the CEBPA promoter separated by 2% agarose gel elec-
trophoresis and visualized with ethidium bromide. Patient sample 6
shows a positive MSP product, indicating CEBPA promoter hyperme-
thylation. Ma: marker, numbers 1-11: patients’ samples, U:
unmethylated positive control, M: methylated positive control, C:
control bisulfites-unreated DNA, B: blank distilled H₂O.
TAD2\(^{20,23}\) (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 \(\text{CEBPA}\).

**Characteristics of \(\text{CEBPA}\) 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. \(\text{CEBPA}\) double mutations were not present in patients below the age of 3 years. However, the median age of patients with \(\text{CEBPA}\) double-mutant AML (12.3 years) did not differ significantly from that of patients with \(\text{CEBPA}\) single-mutant AML (7.5 years) or with \(\text{CEBPA}\) wild-type AML (9.7 years) \((P=0.26)\). \(\text{CEBPA}\) 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 \(\text{CEBPA}\) mutations did not occur in the favorable cytogenetic subgroups [inv(16), t(8;21) and t(15;17)] or in the ABL1-rearranged subgroup. \(\text{CEBPA}\) double mutations occurred mainly in cytogenetically normal AML (57%), but five cases (56%) 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. \(\text{CEBPA}\) 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 \(\text{CEBPA}\) single- and double-mutants, and consisted of FLT3/ITD, RAS and \(\text{WTI}\) mutations, as described in Table 1.

**Identification of one case with gain of a single \(\text{CEBPA}\) mutation at relapse**

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

**Frequency of germ-line origin of \(\text{CEBPA}\) mutations**

Of 7 patients with \(\text{CEBPA}\)-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 \(\text{CEBPA}\) double-mutant AML, the N-terminal frame shift mutation (c.69dupC) was detected in the germ-line material; the second \(\text{CEBPA}\) 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 \(\text{CEBPA}\) 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 \(\text{CEBPA}\) double-mutant cases, indicating that, based on gene expression profiles, the leukemia of this patient was comparable with ‘sporadic’ \(\text{CEBPA}\) double-mutant AML.

**Prognostic impact of \(\text{CEBPA}\) single and double mutations in pediatric acute myeloid leukemia**

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

<p>| Table 1. Characteristics of the 252 pediatric AML patients included in this study, divided by (\text{CEBPA}) mutation status. |
|-------------------------------------------------|-----------------|-----------------|-----------------|-----------------|-----------------|</p>
<table>
<thead>
<tr>
<th>Number</th>
<th>(\text{CEBPA}) single mutation</th>
<th>(\text{CEBPA}) double mutation</th>
<th>(\text{CEBPA}) wild-type</th>
<th>(P) value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, median (years)</td>
<td>9.7 (0.26^*)</td>
<td>7.5</td>
<td>12.3</td>
<td>9.7</td>
</tr>
<tr>
<td>Sex (% female)</td>
<td>45.2% (0.56^*)</td>
<td>66.7%</td>
<td>42.9%</td>
<td>44.8%</td>
</tr>
<tr>
<td>WBC (x10(^9)/L), median (range)</td>
<td>42 (0-535)</td>
<td>20 (8-535)</td>
<td>60 (6-388)</td>
<td>41 (0-483)</td>
</tr>
<tr>
<td>FAB classification, n(%)</td>
<td>0.001#</td>
<td>12 (5%) (1(17%))</td>
<td>-</td>
<td>11 (5%)</td>
</tr>
<tr>
<td>M0</td>
<td>27 (11%)</td>
<td>2 (33%)</td>
<td>6 (43%)</td>
<td>19 (9%)</td>
</tr>
<tr>
<td>M1</td>
<td>55 (23%)</td>
<td>1 (17%)</td>
<td>8 (57%)</td>
<td>46 (21%)</td>
</tr>
<tr>
<td>M3</td>
<td>20 (8%)</td>
<td>-</td>
<td>20 (9%)</td>
<td></td>
</tr>
<tr>
<td>M4</td>
<td>60 (25%)</td>
<td>2 (33%)</td>
<td>-</td>
<td>58 (26%)</td>
</tr>
<tr>
<td>M5</td>
<td>57 (24%)</td>
<td>-</td>
<td>-</td>
<td>57 (26%)</td>
</tr>
<tr>
<td>M6</td>
<td>3 (1%)</td>
<td>-</td>
<td>3 (1%)</td>
<td></td>
</tr>
<tr>
<td>M7</td>
<td>8 (3%)</td>
<td>-</td>
<td>-</td>
<td>8 (4%)</td>
</tr>
<tr>
<td>other</td>
<td>1 (0%)</td>
<td>-</td>
<td>1 (0%)</td>
<td></td>
</tr>
<tr>
<td>Unknown</td>
<td>9 (4%)</td>
<td>- 9 (4%)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Karyotype, n(%)</td>
<td>0.048#</td>
<td>27 (11%)</td>
<td>-</td>
<td>27 (12%)</td>
</tr>
<tr>
<td>t(8;21)</td>
<td>26 (10%)</td>
<td>-</td>
<td>-</td>
<td>26 (11%)</td>
</tr>
<tr>
<td>inv(16)</td>
<td>18 (7%)</td>
<td>-</td>
<td>-</td>
<td>18 (8%)</td>
</tr>
<tr>
<td>11q23</td>
<td>49 (19%)</td>
<td>-</td>
<td>-</td>
<td>49 (21%)</td>
</tr>
<tr>
<td>normal</td>
<td>55 (22%)</td>
<td>2 (33%)</td>
<td>8 (57%)</td>
<td>45 (19%)</td>
</tr>
<tr>
<td>other</td>
<td>60 (24%)</td>
<td>3 (50%)</td>
<td>5 (36%)</td>
<td>49 (21%)</td>
</tr>
<tr>
<td>unknown</td>
<td>17 (7%)</td>
<td>1 (17%)</td>
<td>1 (7%)</td>
<td>15 (7%)</td>
</tr>
<tr>
<td>FLT3/ITD, n(%)</td>
<td>(n=253)</td>
<td>52 (21%)</td>
<td>1 (17%)</td>
<td>3 (21%)</td>
</tr>
<tr>
<td>N- or K-RAS, n(%)</td>
<td>(n=251)</td>
<td>52 (21%)</td>
<td>1 (17%)</td>
<td>2 (14%)</td>
</tr>
<tr>
<td>c-KIT, n(%)</td>
<td>(n=251)</td>
<td>17 (7%)</td>
<td>-</td>
<td>17 (7%)</td>
</tr>
<tr>
<td>MLL-PTD, n(%)</td>
<td>(n=244)</td>
<td>5 (2%)</td>
<td>-</td>
<td>5 (2%)</td>
</tr>
<tr>
<td>NPM1, n(%)</td>
<td>(n=247)</td>
<td>18 (7%)</td>
<td>-</td>
<td>18 (8%)</td>
</tr>
<tr>
<td>WT1, n(%)</td>
<td>(n=250)</td>
<td>27 (11%)</td>
<td>2 (33%)</td>
<td>3 (21%)</td>
</tr>
</tbody>
</table>

\*Kruskal-Wallis test; \#Chi-square test; WBC: white blood cell count at diagnosis; FAB: French-American British.
Patients with \textit{CEBPA} double-mutant AML had a significantly better 5-year overall survival compared with those with a single \textit{CEBPA} mutation (5-year probability of overall survival 79±13\% \textit{versus} 25±22\%; \textit{P}=0.04), although the 5-year event-free survival was not significantly different (5-year probability of event-free survival 58±16\% \textit{versus} 30±24\%; \textit{P}=0.16) (Figure 2). In fact, the outcome of patients with \textit{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\%; \textit{P}=0.51 and of event-free survival of 61±9\%; \textit{P}=0.74. Furthermore, patients with \textit{CEBPA} double mutations showed a clear trend to a more favorable outcome than patients with wild-type \textit{CEBPA} after excluding the core-binding factor-AML cases (5-year probability of overall survival 47±5%; \textit{P}=0.07 and of event-free survival 33±4%; \textit{P}=0.06). The impact of additional molecular or cytogenetic aberrations (e.g. FLT3/ITD) on the \textit{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, \textit{NPM1} mutations and FLT3/ITD, showed that the presence of a \textit{CEBPA} double mutation was an independent favorable prognostic factor for overall survival (HR 0.25; \textit{P}=0.04) as well as event-free survival (HR 0.52; \textit{P}=0.03) (Table 2). \textit{CEBPA} single mutations were not included in the multivariate analysis as a factor because of the small number of cases.

\textbf{Aberrant CEBPA promoter hypermethylation in pediatric acute myeloid leukemia}

Methylation-specific PCR could be performed in 237 cases and revealed hypermethylation of the \textit{CEBPA} promoter region in only three cases (1.3\%) (Figure 1B). As expected, \textit{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 \textit{CEBPA}-hypermethylated cases are shown in Table 3. \textit{CEBPA} promoter hypermethylation was also present in the relapse material (n=2) from these patients, demonstrating clonal stability of the hypermethylation pattern.

![Figure 2. Kaplan-Meier survival curves of overall survival and event-free survival according to \textit{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. \textit{CEBPA} single-mutant AML, \textit{CEBPA} double-mutant AML, \textit{CEBPA} wild-type non-core-binding factor (CBF) AML and CBF-AML.](image_url)

\begin{table}[h]
\centering
\begin{tabular}{|l|l|l|l|l|}
\hline
\textbf{Outcome} & \textbf{Variable} & \textbf{Hazard ratio} & \textbf{95\% confidence interval} & \textbf{P} value \\
\hline
\multirow{2}{*}{OS} & Favorable karyotype & 0.11 & 0.04-0.30 & <0.001 \\
& \textit{CEBPA} double mutation & 0.23 & 0.06-0.96 & 0.04 \\
& \textit{NPM1} mutation & 0.43 & 0.17-1.09 & 0.08 \\
& WBC > 50 x 10\(^3\)/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 \\
\hline
\multirow{2}{*}{EFS} & Favorable karyotype & 0.27 & 0.15-0.47 & <0.001 \\
& \textit{NPM1} mutation & 0.29 & 0.13-0.69 & 0.005 \\
& \textit{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 x 10\(^3\)/L & 1.03 & 0.68-1.54 & 0.90 \\
\hline
\end{tabular}
\caption{Results of multivariate analysis for overall survival (OS) and event-free survival (EFS).}
\end{table}

\textit{WBC}: white blood cell count; \textit{OS}: overall survival; \textit{EFS}: event-free survival.

\textbf{Unsupervised analysis reveals clustering of CEBPA mutant and hypermethylated cases}

Unsupervised cluster analysis of 237 children with \textit{de novo} AML showed distinct clusters (Figure 3A). Cases with \textit{CEBPA} mutations and promoter hypermethylation predominantly clustered together, and are referred to as the main \textit{CEBPA} cluster. This cluster contained 15 cases in total, including eight double-mutants and all three hypermethylated cases. Of interest, two cases of \textit{CEBPA} single-mutant AML were also present in this main \textit{CEBPA} cluster, despite the fact that these cases are expected to have wild-type expression of C/EBP\(\alpha\) p42, in contrast to \textit{CEBPA} double-mutant and hypermethylated cases. Interestingly, extremely high \textit{TRIB2} expression (probe set 202478\_at) was present in one of these \textit{CEBPA} single-mutant cases (#5041), which may explain the C/EBP\(\alpha\) p42-inhibition of its remaining allele, as \textit{TRIB2} directly inactivates C/EBP\(\alpha\) p42.\(^{24}\)

Furthermore, the two remaining cases in the \textit{CEBPA} main cluster had low \textit{CEBPA} gene expression, and clustered closely with the three cases of \textit{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 \textit{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).

<table>
<thead>
<tr>
<th>ID</th>
<th>MSP*</th>
<th>Age (years)</th>
<th>Sex</th>
<th>WBC (x10^9/L)</th>
<th>FAB</th>
<th>Karyotype</th>
<th>Molecular aberration</th>
<th>Treatment protocol</th>
<th>Follow-up</th>
</tr>
</thead>
<tbody>
<tr>
<td>#4736</td>
<td>8,5</td>
<td>female 2.9</td>
<td>237</td>
<td>AM L</td>
<td>NA</td>
<td>46,XX</td>
<td>none</td>
<td>DCI097</td>
<td>relapse after 7.3 months</td>
</tr>
<tr>
<td>#3496</td>
<td>11,3</td>
<td>female 196</td>
<td>237</td>
<td>AM L</td>
<td>M5</td>
<td>NA</td>
<td>none</td>
<td>DCI097</td>
<td>relapse after 8.2 months</td>
</tr>
<tr>
<td>#5033</td>
<td>10,3</td>
<td>female NA</td>
<td>237</td>
<td>AM L</td>
<td>NA</td>
<td>46,XX</td>
<td>none</td>
<td>LAME</td>
<td>CCR for 8.5 years (stem cell transplant 8 months after diagnosis)</td>
</tr>
<tr>
<td>#4728</td>
<td>9,7</td>
<td>male 2.4</td>
<td>237</td>
<td>AM L</td>
<td>NA</td>
<td>46,XX</td>
<td>none</td>
<td>DCI097</td>
<td>relapse after 38.4 months</td>
</tr>
<tr>
<td>#4736</td>
<td>8,5</td>
<td>female 2.9</td>
<td>237</td>
<td>AM L</td>
<td>M4</td>
<td>46,XX</td>
<td>none</td>
<td>DCI097</td>
<td>relapse after 7.3 months</td>
</tr>
</tbody>
</table>

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

**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.\(^{13}\) 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 CD3 weakly (#5033), but expression of other T-cell antigens was not seen. However, high mRNA expression of CD3 (CD32: 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),\(^{13}\) 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.
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. 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 falsely positively recognized (Online Supplementary Table S6B, Online Supplementary Figure S2C,D). This resulted in a low sensitivity (60%) of the 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%). And the two available pediatric series from Taiwan (6%) and North-America (4.5%). 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. 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. 26,28 Single CEBPA mutations are, however, believed to predispose the pre-leukemic initiating cell to subsequent acquisition of secondary (epi)genetic mutations necessary before the development of full-blown AML. 27,28 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, 7 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-mutant 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, 27 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). 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, 41 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. 21 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. Interestingly, two CEBPA single-mutant cases, which are expected to have full-length C/EBPα p42 expression of the unaffected allele, are 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. Complete C/EBPα p42 inactivation of the wild-type allele is hereby established and clustering with cases of AML without functional C/EBPα 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 uni-allelic series based on a prediction signature derived from childhood AML. 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.23 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

The information provided by the authors about contributions from persons listed as authors and in acknowledgments is available with the full text of this paper at www.haematologica.org.

Financial and other disclosures provided by the authors using the ICMJE (www.icmje.org) Uniform Format for Disclosure of Competing Interests are also available at www.haematologica.org.

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