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Combined testing for CCAAT/enhancer-binding protein alpha (CEBPA) mutations and promoter methylation in acute myeloid leukemia demonstrates shared phenotypic features

Philippe Szankasi[a], Albert K. Ho[b], David W. Bahler[b], Olga Efimova[b], Todd W. Kelley[b,*]

[a] ARUP Institute for Clinical and Experimental pathology, ARUP Laboratories, Salt Lake City, UT, USA
[b] Department of Pathology, University of Utah, Salt Lake City, UT, USA

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ABSTRACT

Loss of function mutations in CCAAT/enhancer binding protein alpha (CEBPA) have been identified in acute myeloid leukemia (AML) and bi-allelic (double) *CEBPA* mutations are associated with improved prognosis in cases of cytogenetically normal-AML. In a subset of AML patients lacking *CEBPA* mutations, core promotor methylation of *CEBPA* has been described and is associated with a gene expression profile similar to the mutated cases including the expression of T cell associated genes such as CD7. However, the overall incidence and pattern of *CEBPA* mutations and core promoter methylation has not been thoroughly explored in a larger subset of AML with expression of CD7. Here we describe a simple and clinically deployable *CEBPA* promoter methylation test and the results of combined testing for *CEBPA* mutations and promoter methylation in 102 cases of AML, including 43 CD7+ cases. Overall, there were 5 methylated cases, 6 cases with double mutations, and 3 cases with single mutations. Significantly, 10 of 43 CD7+ cases (23%) had either methylated or double-mutated *CEBPA*. The CD7+ subset included all 5 methylated cases and 5 of the 6 cases with double mutations. All 3 cases with single mutations were CD7−. No case exhibited both hypermethylation and mutations. We find that promoter methylation accounts for half of those CD7+ cases with *CEBPA* dysregulating abnormalities. Furthermore, methylated cases and those with bi-allelic *CEBPA* mutations have similar phenotypic features including expression of CD7 and lack of co-incident *NPM1* mutations. Our study suggests that methylation testing may be as important as mutation testing for identifying AML cases with CEBPA dysregulation and may be indicated in the routine prognostic workup of AML.

1. Introduction

Acute myeloid leukemia (AML) is a heterogeneous disease both clinically and genetically and prognostic categories based on recurrent cytogenetic findings and/or the presence of recurrent gene mutations in *NPM1*, *FLT-3* and, more recently, the transcription factor CCAAT/enhancer-binding protein alpha (CEBPA) have been defined. *CEBPA* is located at chromosome 19q3.1, is expressed as high levels in granulocyte/monocyte progenitor (GMP) cells, and appears to play an important role in myeloid differentiation and suppression of proliferation [1,2]. *CEBPA* mutations occur in a subset of cases of AML, usually in the context of cytogenetically normal-AML (CN-AML) patients [3]. *CEBPA* mutations and *NPM1* mutations are usually mutually exclusive [3,4] and this finding provides biologic evidence for a distinct mechanism of leukemogenesis among these cases. Accordingly, AML with *NPM1* mutations and AML with *CEBPA* mutations are included as separate provisional entities in the 2008 WHO classification of hematopoietic neoplasms [5]. *CEBPA* mutations and *FLT-3* internal tandem duplication (ITD) mutations do co-exist in a small subset of cases and outcomes in these patients appear significantly worse [6]. Germline heterozygous mutations in *CEBPA* have recently been described in which a sporadic *CEBPA* mutation on the opposite allele is detected in those who go on to develop AML [7]. The germline mutations that have been described are N-terminal single nucleotide insertions resulting in an out-of-frame sequence [7,8]. These should not be confused with the described in-frame germline polymorphisms that also occur in the gene [9].

Mutations in *CEBPA* confer a more favorable outcome but this prognosis appears to be associated with only bi-allelic (double) mutations [4,10,11]. Mutations predominantly occur in two general regions, either in one of the two N-terminal transcription activation domains (TADs) or in a C-terminal basic domain-leucine zipper (bZIP) that mediates DNA binding and homo/hetero-dimerization.

* Corresponding author at: Department of Pathology, University of Utah, 15 North Medical Drive East, Room 2100, Salt Lake City, UT 84054, USA. Tel.: +1 801 213 2389; fax: +1 801 585 3831.
E-mail address: todd.kelley@path.utah.edu (T.W. Kelley).

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However, this is not always the case and numerous mutations, both insertions/deletions and substitutions, have been described throughout the gene. Patients with CEBPA mutations usually exhibit one TAD mutation and one b-ZIP mutation but occasional variations in the pattern are also described. The significance of double *CEBPA* mutations appear mostly resolved but the biologic and prognostic meaning of a single *CEBPA* mutation remains uncertain. It also remains to be seen whether or not less common mutation patterns (i.e. 2 C-terminal mutations or 2 N-terminal mutations) are of clinical and/or biologic relevance.

The presence of epigenetic perturbations in AML including over/under expression of regulatory micro RNAs (miRNA) and specific gene methylation profiles appear important in the pathophysiology of the disease [12]. In line with this, *CEBPA* silencing through promoter methylation by DNA methyltransferases may also be an important event in leukemogenesis [13,14]. There are two regulatory regions upstream of the *CEBPA* transcription start site, a core promoter region located at approximately [0 to −300] and an upstream regulatory region beginning at approximately [−1121] [15]. The upstream regulatory region is methylated in solid tumors [16,17] and, interestingly, in a large percentage of AML cases and is associated with the presence of t(15;17) and inv(16) cytogenetic abnormalities [15]. The core promoter region is hypermethylated in a subset of AML cases with reported ranges from <1% to 12% of cases [13,14]. Furthermore, cases with *CEBPA* core promoter hypermethylation co-cluster with *CEBPA*-mutated cases in gene expression profiling studies [14]. This finding indicates that core promoter methylation and double mutations may confer similar biologic effects on the tumor cells most likely arising from a lack of functional CEBPA protein. An additional feature of cases with core promoter methylation is aberrant expression of T cell associated genes such as *LCK* and *CD7* [14]. CD7 expression has also been observed frequently in *CEBPA*-mutated cases [18]. However, the incidence and pattern of total *CEBPA* dysregulating events, including methylation, in AML with and without CD7 expression is uncertain.

Overall, the pathophysiologic and prognostic significance of *CEBPA* promoter methylation has not been well characterized in AML. In this study, we analyzed methylation status of the *CEBPA* core promoter region given the previously shown biologic similarities between AML with core promoter methylation and those cases with *CEBPA* mutations. Our goals were to develop a rapid and efficient *CEBPA* promoter methylation assay that would be well suited for a clinical hematology laboratory. Further, we wanted to evaluate the frequency of promoter methylation in a series of AML samples that was enriched for cases demonstrating CD7 expression, and to analyze the associations between *CEBPA* methylation and *CEBPA* mutation status (both single and double mutations) with blast immunophenotype by flow cytometry and with other available clinical parameters such as the presence of *NPM1* exon 12 mutations.

2. Materials and methods

2.1. Patient samples, flow cytometry, DNA extraction

Excess cryopreserved whole blood or bone marrow aspirate-derive white blood cells were obtained from the ARUP hematologic flow cytomery laboratory after complete clinical flow cytometric evaluation. The use of these de-identified patient specimens was approved by the University of Utah Institutional Review Board (IRB# 7275). Samples were subjected to flow cytometric immunophenotyping as previously described in Ref. [19]. Positivity by flow cytometry was defined as expression in at least 20% of cells in the gated population of interest, compared to internal negative control cells. All samples were reviewed by a hematopathologist and were diagnostic of acute myeloid leukemia based on morphology and immunophenotype and all contained at least 20% leukemic blasts. A total of 43 CD7 positive cases were available for analysis. For comparison purposes, an additional 59 CD7 negative cases were then randomly selected from the archive. Cells were harvested by centrifugation and genomic DNA was extracted using the Cultured Cells protocol of the Puregene Kit (Qiagen, Inc., Valencia, CA, catalog number 158745). DNA samples were brought to a final concentration of 50 ng/μL. Karyotypic data was not available on any of the cases.

Table 1
Primers.

Primer	Sequence (5′–3′)
CEBPA-1F	PET-5′-ATGCCGGGAGAACTCTAAC
CEBPA-1R	5′-CGCGCCCGGGTAGTCAAAGTC
CEBPA-2F	5′-AGCAGGAGAAGGCCAAGGCG
CEBPA-2R	FAM-5′-TGGTGGTCTGGCCGCAGTGC
CEBPA-3F	NED-5′-CGCACCTGCAGTTCCAGATC
CEBPA-3R	5′-CTCGTTGCTGTTCTTGTCC
CEBPA-4F	5′-GGGCAAGGCCAAGAAGTC
CEBPA-4R	VIC-5′-GGCTGGCCCAGGGCGGT
CpG-F	biotin-5′-TTGGAGG(C/T)GGTGGG(C/T)GTT
CPG-R	5′-CCCACCC(G/A)AAAACCCTACT
CPGseq	5′-CCTACTAAATCCTAAC
CEBPAq-F	5′-ACGTGGAGACGCAGCAGAAG
CEBPAq-R	5′-AAGGAGCTCTCTGGCAGCTG

2.2. Mutation analysis

The *CEBPA* coding region was PCR amplified on four overlapping fragments (fragments 1 through 4) with primers listed in Table 1. Each PCR reaction contained 100 ng genomic DNA, Phusion DNA Polymerase Mastermix with GC Buffer (New England Biolabs, Waltham, MA), 5% (v/v) DMSO, and 0.3 μM each primer (CEBPA-1F and CEBPA-1R for fragment 1, or CEBPA-2F and CEBPA-2R for fragment 2, or CEBPA-3F and CEBPA-3R for fragment 3, or CEBPA-4F and CEBPA-4R for fragment 4) in a final volume of 20 μL. PCR reactions were pre-incubated at 98 °C for 30 s followed by 10 cycles of 98 °C for 10 s, 72 °C (−1 °C per cycle) for 10 s, and 72° for 15 s, followed by 35 cycles of 98 °C for 10 s, 62 °C for 10 s, and 72 °C for 15 s, followed by a 2-min hold at 72 °C and a cool down. The PCR reactions for fragments 1, 2, 3, and 4 were diluted in water 5, 2, 20, and 40-fold, respectively. Equal volumes of the diluted PCR fragments were combined and 1 μL was mixed with 8.5 μL HiDi formamide (Applied Biosystems, Inc., Foster City, CA) and 0.5 μL GeneScan LIZ-500 internal size standards (Applied Biosystems, Inc.) and heated to 95 °C for 2 min. The samples were run on an ABI 3100 Genetic Analyzer using 50-cm capillaries with POP-6 polymer. The samples were injected at 3 kV for 10 s and run at 15 kV for 5500 s at 60 °C. PCR products and internal standards were detected using filter set G5. Raw data were analyzed with GeneMapper v4.0 software (Applied Biosystems, Inc., Foster City, CA). Samples with aberrant DNA fragment length were confirmed by DNA sequence analysis. For this, the *CEBPA* coding region was PCR amplified on two overlapping fragments using the unlabeled version of the primer pairs CEBPA-1F and CEBPA-2R, and CEBPA-3F and CEBPA-4R. The PCR reactions contained 100 ng genomic DNA, 1.5 units GoTaq Flexi DNA polymerase (Promega Corp., Madison, WI), GoTaq Flexi buffer (Promega), 5% (v/v) DMSO, 3 mM $MgCl_2$, 0.2 mM each dNTP, and 0.2 μM each primer in a final volume of 20 μL. The reactions were incubated at 94 °C for 2 min, followed by 10 cycles of 94 °C for 30 s, 70 °C (−1° each cycle) for 20 s, and 72 °C for 10 s (+2 s each cycle) followed by 35 cycles of 94 °C for 20 s, 60 °C for 20 s, and 72 °C for 30 s, followed by a 2-min hold at 72 °C and a cool down. Five microlitre of the reactions were analyzed on a 2% agarose gel and visualized with ethidium bromide under UV light. For direct DNA sequence analysis 5 μL of the PCR reactions were mixed with 1 μL ExoSAP-IT (USB Corp., Cleveland OH) and incubated at 37 °C for 45 min followed by heat inactivation for 5 min at 85 °C. After appropriate dilution in water, 6 μL of each sample was combined with 8 μL of 0.8 μM forward or reverse PCR primer and sequenced using BigDye terminator technology (Applied Biosystems, Inc., Foster City, CA) and analyzed on an ABI3730 Genetic Analyzer according to the manufacturer's instructions. *NPM1* mutation analysis was performed as previously described in Ref. [20].

2.3. CpG methylation analysis

For CpG methylation analysis, 500 ng of genomic DNA was bisulfite converted with the EZ DNA Methylation Kit (Zymo Research, Orange, CA) according to the manufacturer's instructions, except that the final elution volume was 20 μL. Four microlitre of the bisulfite converted DNA were PCR amplified in a final volume of 25 μL containing 1.5 units HotStart Taq DNA Polymerase (Qiagen, Inc., Valencia, CA), PCR buffer (Qiagen Inc.), 3 mM (final) $MgCl_2$ (1.5 mM $MgCl_2$ is contributed by the PCR buffer), 0.2 mM each dNTP (stock solution stored at 4 °C), 0.2 μM biotinylated forward primer CpG-F, 0.2 μM reverse primer CpG-R (Table 1). PCR reactions were pre-incubated at 94 °C for 15 min followed by 10 cycles of 94 °C for 20 s, 70 °C (−1 °C every cycle) for 20 s, and 72 °C for 10 s (+1 s every cycle), followed by 35 cycles of 94 °C for 20 s, 60 °C for 20 s, and 72 °C for 20 s, followed by a 2-min hold at 72 °C and a cooldown. Four microlitre of the PCR reaction were run on a 2% agarose gel, and DNA was visualized by ethidium bromide (0.5 μg/mL) and UV light. From the remaining DNA the biotinylated forward strand was isolated and sequenced on the PyroMark Q24 pyrosequencer according to the manufacturer's instructions. From the sequencing primer CpG-seq (Table 1) the sequence 5′-RCR CRA CCR ACA TAA