

The *CEBPA* gene is down-regulated in acute promyelocytic leukemia and its upstream promoter, but not the core promoter, is highly methylated

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ABSTRACT

Impairment of CCAAT Enhancer Binding Protein alpha (CEBPA) function is a common finding in acute myeloid leukemia; nevertheless, its relevance for acute promyelocytic leukemia pathogenesis is unclear. We analyzed the expression and assessed the methylation status of the core and upstream promoters of *CEBPA* in acute promyelocytic leukemia at diagnosis. Patients with acute promyelocytic leukemia (n=18) presented lower levels of *CEBPA* expression compared to healthy controls (n=5), but higher levels than those in acute myeloid leukemia with t(8;21) (n=9) and with inv(16) (n=5). Regarding the core promoter, we detected no methylation in 39 acute promyelocytic leukemia samples or in 8 samples from controls. In contrast, analysis of the upstream promoter showed methylation in 37 of 39 samples, with 17 patients showing methylation levels over 30%. Our results corroborate data

obtained in animal models showing that *CEBPA* is down-regulated in acute promyelocytic leukemia stem cells and suggest that epigenetic mechanisms may be involved.

Key words: CEBPA, acute promyelocytic leukemia, DNA methylation.

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Introduction

Partial loss of function of the transcription factor CCAAT enhancer binding protein alpha (CEBPA) is a common finding in acute myeloid leukemia (AML). The *CEBPA* gene is found to be mutated in approximately 5-14% of acute myeloid leukemia patients.¹ Moreover, *CEBPA* expression is repressed by the RUNX1-RUNX1T1 oncoprotein associated with acute myeloid leukemia with t(8;21). Wouters *et al.*,² studying acute myeloid leukemia cases with a silenced non-mutated *CEBPA* gene, reported the hypermethylation of the proximal promoter (-428 to +64 relative to the transcription start site, TSS). In addition, Figueroa *et al.*³ analyzed the same acute myeloid leukemia subset and detected higher DNA methylation in two exonic regions (+1334 to +1980 and +857 to +1113) and in two upstream promoter regions (-1569 to -1819 and -2247 to -2863).

The downregulation of *CEBPA* in acute promyelocytic leukemia (APL) is controversial. Pabst *et al.*⁴ and Cilloni *et al.*⁵ did not detect significant differences in acute promyelocytic leukemia compared to normal bone marrow (NBM) samples. In contrast, Sukhai *et al.*⁶ reported lower expression in 11 of 12 acute promyelocytic leukemia patients. Moreover, treatment of cell lines expressing PML-RARA with a DNA methylase

inhibitor, but not with a histone deacetylase inhibitor, induced *CEBPA* expression.⁷ Recently, we isolated and characterized the APL-initiating cell population in bone marrow of a murine model of acute promyelocytic leukemia and detected markedly reduced levels of *CEBPA* mRNA and protein in this subset compared with promyelocytes isolated from wild-type mice.⁷

In the present study, we analyzed *CEBPA* expression and the methylation status of the core promoter and of a recently identified upstream promoter in 39 patients with acute promyelocytic leukemia.

Design and Methods

Patient samples, total RNA and genomic DNA isolation

Total RNA from bone marrow mononuclear cells (BMMCs) of 18 patients with acute promyelocytic leukemia, 14 patients with acute myeloid leukemia, including 9 with t(8;21) and 5 with inv(16) at diagnosis, and of 5 healthy individuals were isolated using Trizol (Invitrogen) following the manufacturer's recommendations. Genomic DNA was isolated from bone marrow mononuclear cells of 39 acute promyelocytic leukemia patients at diagnosis and from peripheral blood mononuclear cells (PBMCs) of 8 healthy controls using the Puregene DNA Purification Kit (Gentra Systems) according to the manufacturer's instructions. Samples were collected after obtaining

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informed consent under a protocol approved by the Medical School of Ribeirão Preto, University of São Paulo.

Analysis of PML breakpoint regions and FLT3-ITD mutations

The *PML* breakpoint region [on intron 6 (bcr 1), intron 3 (bcr 3) or exon 6 (bcr 2), thus generating different *PML-RARA* isoforms] was determined by RT-PCR, as previously described.⁸ The screening for Internal Tandem Duplication mutations in the *FLT3* gene (*FLT3-ITD*) was performed on genomic DNA by PCR, as previously described.⁹

Expression analysis by real-time PCR

One μg of total RNA was incubated with 2 units of *DNase I* (Invitrogen) for 30 min at room temperature. The DNA-free RNA was reverse transcribed using a High Capacity cDNA Reverse Transcription Kit (Applied Biosystems). Relative expression of the *CEBPA* gene (probe Hs00269972_s1; Applied Biosystems) was determined by the $2^{-\Delta\Delta\text{CT}}$ method [ΔCT of a sample with t(8;21) was used as reference] and the housekeeping gene transcript *Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)*; probe 4310884E-0502026; Applied Biosystems) was used to normalize the results in a 7300 real-time PCR System (Applied Biosystems).

Methylation-specific polymerase chain reaction (MSP) and bisulfite sequencing

Bisulfite modification of DNA was carried out with the EZ DNA Methylation Kit (Zymo Research) according to the manufacturer's recommendations. The treatment of DNA with bisulfite results in selective conversion of unmethylated cytosine to uracil, whereas methylated cytosine remains unchanged. The following primers were used for methylation-specific polymerase chain reaction of the core promoter of the *CEBPA* gene (positions of methylated-M and unmethylated-U primers: -286 to -68 and -288 to -67 bp from the TSS, respectively): M-*CEBPA* Fw: 5'-GGGTTTTTCGTTATC-GATTACGT-3' and M-*CEBPA* Rv: 5'-CCTCTATCCCCGAAC-TACGA-3'; U-*CEBPA* Fw: 5'-GGGGTTTTTGTTATTGATTAT-GT-3' and U-*CEBPA* Rv: 5'-ACCTCTATCCCCAAACTACA-3'. The primers were designed by the MethPrimer program¹⁰ using the promoter sequence of *CEBPA* (gene accession number: U34070.1). For bisulfite sequencing of the upstream promoter of the *CEBPA* gene we used Fw: 5'-TTGTTAGGTTAAGGT-TATTG-3' and Rv: 5'-AAACCCTAAAACCCCTTA-3' (position -1422 to -1121 bp from the TSS). PCR products were subcloned into pCR4-TOPO (Invitrogen), transformed bacteria were cultured overnight and the plasmid DNA was isolated using the SNAP Miniprep Kit (Invitrogen) and sequenced. Three independent clones were sequenced for each sample using the ABI BigDye Terminator Cycle Sequencing Kit (Applied Biosystems). For this study, the methylation at an individual CpG site (out of 25 CpG) was identified using the sequencing analysis tools from the CLC Main Workbench Software 5 (CLC bio) and counted in all clones, and an average methylation percentage was calculated for each patient's sample.

Western blotting

Protein extracts obtained from Trizol (Invitrogen) were equally loaded on 12.5% homogeneous SDS-PAGE gel and electro-blotted to a PVDF membrane (Hybond-P, GE Healthcare). The PVDF membrane was blocked with 5% non-fat milk in TBS-T. Blots were incubated with the primary polyclonal antibody anti-C/EBP α (Cell Signaling) and primary monoclonal antibody anti-B23 (Nucleophosmin/NPM) (Sigma) at 1:1,000 dilutions at 4°C for 16-18 h, followed by 1-hour incubation with secondary antibody (anti-rabbit) (Cell Signaling) or anti-mouse (Sigma) immunoglobu-

lin (1:2,000). Blot signals were detected by enhanced chemiluminescence (GE Healthcare) on X-ray film.

Statistical analysis

CEBPA expression and upstream promoter region methylation levels were compared between patient samples and samples from healthy donors using analysis of variance (ANOVA) followed by Bonferroni's test and the unpaired t-test, respectively (GraphPadPrism 5 software).

Results and Discussion

CEBPA gene expression in AML samples

Figure 1 shows *CEBPA* gene expression in 32 acute myeloid leukemia and 5 normal bone marrow samples. In agreement with previous studies^{4,5,11} acute myeloid leukemia cases with t(8;21)/RUNX1-RUNX1T1 and inv(16)/CBFB-MYH11 (core-binding factor leukemias, CBF) presented similar levels, which were significantly lower than those of the normal group. Since our aim was to evaluate *CEBPA* expression in acute promyelocytic leukemia, we selected the CBF acute myeloid leukemia as a bona fide example of its downregulation. The mean value of *CEBPA* expression in acute promyelocytic leukemia patients was intermediate between those detected in CBF leukemias and in normal bone marrow. Nevertheless, due to the small number of patients analyzed this result should be interpreted cautiously.

Our results agree with those of Sukhai *et al.*⁶ but contrast with those previously reported by Pabst *et al.*⁴ and Cilloni *et al.*⁵ However, there are important methodological considerations. Pabst *et al.*⁴ used a competitive quantitative reverse-transcriptase (RT)-PCR assay, and Cilloni *et al.*⁵ used real-time PCR and normalized the *CEBPA* copy number to 10,000 copies of the *ABL* constitutive gene. Nevertheless, by reviewing the expression values shown in the previous reports and comparing them to our results it is possible to state that *CEBPA* expression is down-regulated in acute promyelocytic leukemia although to a lesser extent than in CBF leukemias.

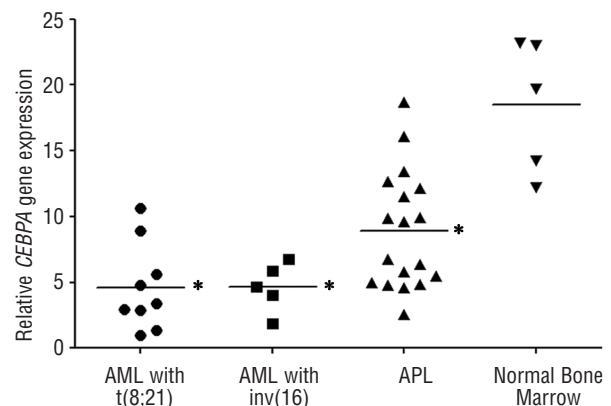


Figure 1. Relative expression of *CEBPA* gene in samples from leukemic patients with t(8;21) (n=9), inv(16) (n=5), t(15;17) (n=18) and from healthy controls (n=5, normal bone marrow). ΔCT of a sample with t(8;21) was used as reference for real-time PCR analysis and *GAPDH* was used as the internal control gene. * Significantly lower expression compared to healthy controls ($P < 0.05$).

Twelve out of 39 acute promyelocytic leukemia patients presented FLT3-ITD mutation. *CEBPA* expression levels were evaluated in 18 patients and no significant difference was detected between FLT3 mutated (n=8) and non-mutated (n=10) cases (9.8 ± 4.9 vs. 8.1 ± 4.2 , respectively). Similarly, no difference was detected between acute promyelocytic leukemia cases harboring the bcr1 (56.5%) or bcr3 (43.5%) *PML-RARA* isoforms (data not shown).

DNA methylation status analysis of the CpG islands in the core and upstream promoters of *CEBPA* in APL

To investigate if *CEBPA* mRNA downregulation observed in acute promyelocytic leukemia was associated with the methylation status of the core and/or upstream promoter regions of *CEBPA*, we used the MSP and bisulfite sequencing methods. None of the 39 acute promyelocytic leukemia or of the 8 normal samples showed *CEBPA* methylation in the core promoter, which is in agreement with the studies by Chim *et al.*¹² and Jost *et al.*,¹³ who analyzed 20 and 4 acute promyelocytic leukemia samples, respectively. A major limitation of the MSP technique concerns the restricted span of genomic DNA analyzed. The studies by Chim *et al.*,¹² Jost *et al.*¹³ and this present study amplified approximately the same sequence. Nevertheless, Agrawal *et al.*,¹⁴ using a genome-wide screening strategy (Matrix-assisted laser desorption/ionization time-of-flight mass spectrometry: MALDI-TOF MS) analyzed the region +4 to -531 bp relative to the TSS of the *CEBPA* gene in 81 acute myeloid leukemia cases (including 3 acute promyelocytic leukemia) and found low methylation levels in all samples. In addition, Hackanson *et al.*¹¹ studied 7 acute promyelocytic leukemia patients by MassARRAY and found no methylation in the core promoter. To our knowledge, the present study analyzed the largest cohort of acute promyelocytic leukemia patients and, taken together with existing literature data, it suggests that methylation in the core promoter is not involved in the epigenetic regulation of *CEBPA* expression in acute promyelocytic leukemia.

Epigenetic regulation of *CEBPA* expression through DNA methylation has been demonstrated in lung cancer¹⁵ and head and neck squamous cell carcinoma.¹⁶ Tada *et al.*¹⁵ demonstrated that the interaction of MBD2 and MeCP2 methyl-CpG binding proteins with the upstream promoter regulatory region spanning -1413 to -887 bp from TSS was associated with the decrease of *CEBPA* expression in lung cancer cell lines. In the -1256 to -831 region there are binding sites for the transcriptional factors USF-1/-2 and Sp1, thus suggesting that methylation decreases the cis-activity of these factors, leading to lower *CEBPA* expression.¹⁵

We analyzed the methylation status of the *CEBPA* upstream promoter region in 39 acute promyelocytic leukemia cases by bisulfite sequencing (position -1422 to -1121 bp from TSS). Figure 2A shows that in 37 samples at least one CpG site was methylated (median 22.70%; range 1.3-66.0%). In addition, 17 patients showed more than 30% of the CpG sites methylated, in contrast to samples from healthy donors (median 1.65%; range 0-4%). The CpG sites from 2 to 12 were those most frequently methylated in the patients' samples (median 49.6%, range 40.9-53.9%) (Figure 2B).

Our data agree with recent findings reported by Hackanson *et al.*¹¹ However, these authors reported lower levels of methylation (median 5%) in the same genomic region compared with the results presented here. It should be pointed out that Hackanson *et al.*¹¹ used the BioCOBRA technology (combined bisulfite restriction analysis coupled with the Agilent 2100 Bioanalyzer platform).

We did not detect a direct correlation between *CEBPA* gene expression and aberrant methylation of the upstream promoter region in acute promyelocytic leukemia. However, this finding does not rule out the relevance of epigenetic control of *CEBPA* expression, since other unknown regions may be involved. Indeed, we analyzed protein expression in 2 patients whose blasts presented 0% and 37.3% of the CpG sites methylated, and detected higher *CEBPA* levels in the former (Figure 3). The ability of *PML-*

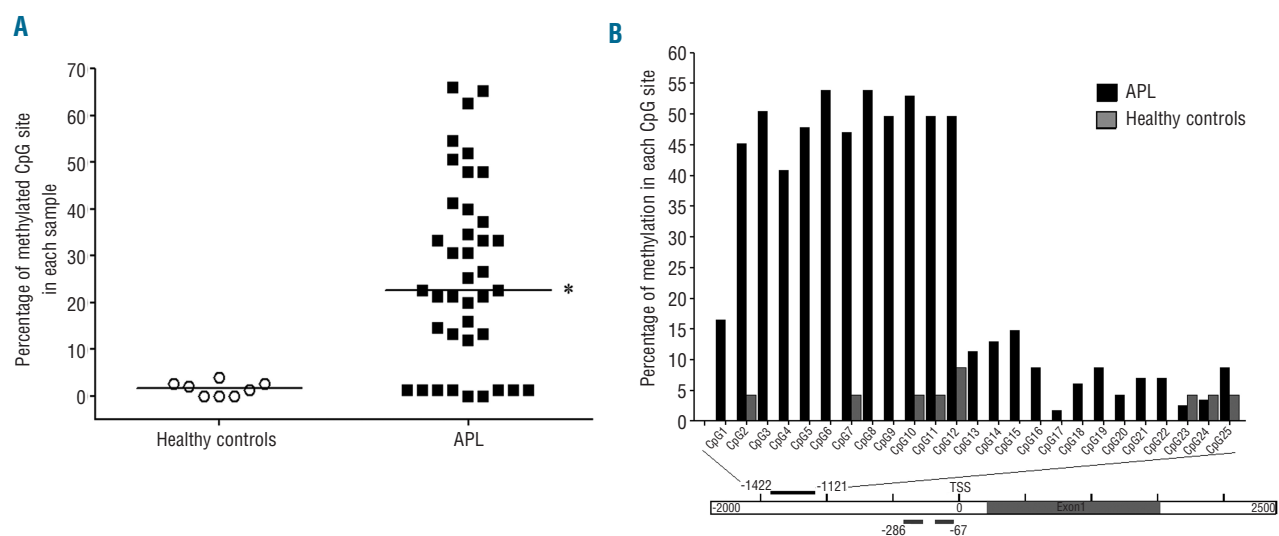


Figure 2. DNA methylation analysis of the *CEBPA* upstream promoter of APL samples. (A) Percentage of methylated CpG sites of 39 APL samples and of 8 samples from healthy controls using bisulfite sequencing. * Median value of the percentage of methylated CpG sites was significantly higher compared to healthy controls at $P=0.0011$. (B) Percentage of methylation at each CpG site of the *CEBPA* upstream promoter in APL samples (black bars) and in samples from healthy controls (gray bars). In the schematic presentation of the *CEBPA* gene below, the areas of DNA methylation analyses by bisulfite sequencing and MSP are indicated as black and gray lines, respectively; TSS: transcription start site.

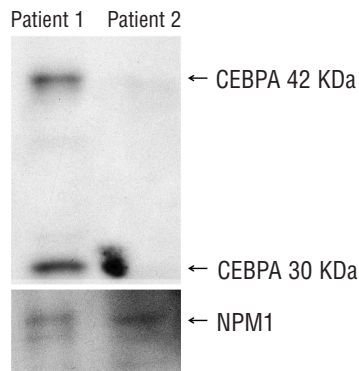


Figure 3. Western blot of CEBPA protein in APL patient samples 1 and 2, which presented 0% and 37.3% of the CpG sites methylated, respectively (upper panel). Equal protein loading was assessed by evaluating NPM1 protein expression (lower panel).

RARA to recruit the corepressor complex harboring histone deacetylase and DNA methylase activity is well known.^{17,18} Guibal *et al.*⁷ showed that treatment of acute promyelocytic leukemia cell lines (NB4 and HT93) with a demethylating agent (5-Aza) resulted in a significant increase of CEBPA RNA and protein levels, while no change was observed with treatment with a histone deacetylase inhibitor (trichostatin A). Expression of the PML-RARA mutant protein, which is unable to recruit the histone deacetylase complex through its N-Cor domain, in the U937 cell line, maintained the CEBPA downregulation observed in the presence of non-mutant PML-RARA protein. Thus, these findings support the hypothesis of CEBPA repression via a DNA methylation pathway.

Although the proximal promoter of CEBPA does not have

any obvious retinoic acid binding site, Guibal *et al.*,⁷ using a PML-RARA conditional expression model, demonstrated that the presence of the fusion protein significantly decreased by 60% the CEBPA-binding activity as a result of lower RNA and protein expression. PML-RARA recruitment to C/EBPA regulatory elements could also occur through an indirect mechanism. Wageningen *et al.*¹⁹ show that PML-RARA physically interacts with Sp1 in the absence of DNA and proposed a model in which PML-RARA binds to a DNA-bound Sp1/NF-Y complex, thus modulating gene expression. The CEBPA upstream methylated region (-1423 to -1121 bp from TSS) contains two potential SP1 and USF binding sites¹¹ that could enable recruitment of PML-RARA.

In conclusion, the CEBPA gene is down-regulated in acute promyelocytic leukemia and its upstream promoter region is highly methylated. However, the mechanisms regulating CEBPA expression in acute promyelocytic leukemia have not been completely identified. Nevertheless, along with the data reported by Guibal *et al.*,⁷ our results support a role for CEBPA deregulation in acute promyelocytic leukemia genesis.

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.

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