Original article

CCAAT/enhancer binding protein α gene expression in Egyptian patients with acute myeloid leukemia

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Abstract Background: Transcription factors play a crucial role in myeloid differentiation and lineage determination. Tumor suppressor protein C/EBPα is a key regulator of granulocytic differentiation whose functional inactivation has become a pathophysiological signature of myeloid leukemia. Given the role that CCAAT/enhancer binding protein α (C/EBP α) plays in myelopoiesis, we anticipated that their expression might be disrupted in myeloid neoplasms.

Purpose: To estimate the expression of C/EBPα mRNA in patients with acute myeloid leukemia and correlate its expression with the pathogenesis of the disease.

Patients and methods: Forty AML patients and 20 age and sex matched healthy controls were included in the study. Blood samples of patients and controls were analyzed for CEBPα mRNA expression by quantitative RT-real time PCR using TaqMan technology & ΔΔCt method for calculation of gene expression.

Results: Twenty-nine (72.5%) patients out of the 40 showed low expression levels of CEBPα mRNA below the cutoff value with median of 0.19 (range:0–0.87). While eleven (27.5%) patients out of the 40 showed higher expression levels of CEBPα above the cutoff value with median of 1.52 (range:1.07–2). Seven patients out of the 11 showed higher expression levels of CEBPα mRNA belong to the M3 subtype of AML harboring the t(15;17) PML–RARα translocation.
Introduction

Acute myeloid leukemia is the most frequent hematological malignancy in adults, with an estimated worldwide annual incidence of three to four cases per 100,000 people. Despite intensive research for new therapies and prognostic markers, it is still a disease with a highly variable prognosis among patients and a high mortality rate. Indeed, less than 50% of adult AML patients have a 5-year overall survival rate (OS), and, in the elderly, only 20% survive 2 years [1].

Acute myeloid leukemia (AML) is a cytogenetically and molecularly heterogeneous disease characterized by clonal proliferation of myeloid precursors with maturation arrest of myeloid cells in the bone marrow and impaired production of normal blood cells. Thus, the leukemic cell infiltration in marrow is accompanied, nearly invariably, by anemia and thrombocytopenia. The absolute neutrophil count may be low or normal, depending on the total white cell count [2].

C/EBPs are a family of transcription factors that regulate cell growth and differentiation. These factors, particularly C/EBPa and C/EBPβ, have important roles in normal hematopoiesis [3].

C/EBPα is the founding member of a family of basic region/leucine zipper (bZIP) transcription factors and is a master regulator of granulopoiesis. It is expressed at high levels throughout myeloid differentiation and binds to the promoters of multiple myeloid-specific genes at different stages of myeloid maturation [5]. C/EBPs is probably part of a self-reinforcing pathway that causes myeloid cells to become postmitotic and to assume the characteristics of mature neutrophils [4].

Target genes of C/EBPα include granulocyte colony stimulating factor (G-CSF) receptor and myeloperoxidase. In addition, C/EBPα is able to block growth by enhancing the activity of the cyclin-dependent kinase (cdk) inhibitor p21, inhibiting E2F transactivation, and blocking cdk2 and cdk4 activity [6,7].

Interaction of transcription factors with other nuclear proteins plays an important role in a combinatorial fashion in stem cell development, lineage commitment and differentiation in the hematopoietic system. Besides mutations, C/EBPα protein function is also disrupted by negative protein–protein interactions in AML. In many of those AML cases that are associated with chromosomal translocation, the resulting translocation product disrupts the expression and function of the same lineage factor and the regulatory proteins key to the maintenance of normal hematopoiesis. C/EBPα, being the key regulator of granulopoiesis, seems to be easily disrupted by these translocations [8,9].

For example, the PML–RARA fusion protein, signature of acute promyelocytic leukemia (APL), results from the translocation t(15;17) blocking promyelocytic differentiation [10]. In primary human APL cells, PML–RARA inhibits C/EBPα function by down regulating its expression. Also, t(15;17) post-translationally inhibits C/EBPα DNA binding activity via a mechanism that involves the formation of the PML/RARA-C/EBPα protein complex. Moreover, over-expression of C/EBPα in acute promyelocytic leukemia cells antagonizes the differentiation-inhibitory effect of PML/RARA [11].

More recently, Tokita and colleagues showed that RUNX1/EVI1 chimeric transcription factor produced by t(3;21) causes leukemic transformation in hematopoietic stem cell tumors, possibly through a differentiation block of malignant myeloid progenitors by associating with C/EBPα and thereby inhibiting its function [12].

AML with the t(8;21) translocation gives rise to the fusion gene AML1–ETO encoding the AML1–ETO fusion protein. AML1–ETO appears to suppress C/EBPα expression indirectly by inhibiting positive autoregulation of the C/EBPα promoter. In addition, conditional expression of C/EBPα overcomes the block of differentiation caused by AML1–ETO and is sufficient to trigger terminal neutrophilic differentiation. Restoring C/EBPα expression will have therapeutic applications in AML1–ETO-positive Leukemias [13].

The previous observations raise the possibility that decreased expression of C/EBPα might have a role in the pathogenesis of AML; hence real time PCR was performed to estimate expression of C/EBPα mRNA in AML patients.

Materials and methods

Participants

After informed patient consent and approval by the Institutional Review Board (IRB), 40 newly diagnosed AML patients referred to Kasr El Aini Centre of Oncology and Radiation (NEMROCK) in the period from 2009 to 2011 were studied. The diagnosis of AML was based mainly on morphological FAB classification. Patients were diagnosed in the Clinical Pathology department and treated in Clinical Oncology department, Kasr Al-Aini, Cairo University. Twenty age and sex matched healthy controls were enrolled in this study. Ethical approval & Informed consent were obtained from all participants.

ATRA can induce a complete remission in most patients with APL. Other FAB subtypes’ patients received induction therapy; 7 + 3 protocol. Three days of an anthracycline: (daunorubicin, at least 60 mg/m2, idarubicin, 10–12 mg/m2, or the anthracyclene mitoxantrone, 10–12 mg/m2), 7 days of cytarabine (100–200 mg/m2 continuous IV).

After a median duration of follow up for 6 months, the overall survival rate: defined from the date of the first visit till the last follow up) and the Disease Free Survival rate (DFS: defined from the date of CR achievement till the date the patient relapsed) were assessed.

Conclusion: We conclude that the majority of the AML patients analyzed, express low levels of C/EBPa mRNA. However, a subset of patients represented by the M3 subtype, express higher levels of C/EBPa.
Methodology

Polymerase chain reaction (PCR)

RNA isolation and cDNA synthesis
Total RNA was extracted from peripheral blood or bone marrow blast cells using commercially available extraction kits (Qiagen, Germany). The amount of RNA was measured spectrophotometrically. The RNA integrity was tested on the Nanodrop. All samples had an OD 260/280 nm ratio > 1.8, indicating high purity.

For the generation of first strand cDNA, 2 μg of total RNA was reversed transcribed in a final volume of 100 μl with the high capacity cDNA Archive Kit (Applied Biosystem, Netherlands).

PCR amplification

cDNA specific CEBPα Taqman primer and probe sets were developed using primer express software. The nucleotide numbering throughout this study is based on the published sequence available from EMBL/GenBank/DDBJ under accession number NM_004364.1: The forward primer of CEBPα (5′-TCGGTTGCAAGAACAG-3′), the reverse primer (5′-GCAGGCCGGTCATT-3′), and the probe (6-FAM)-ACAA GGCCAAGCCAGGC-[TAMRA-6-FAM]).

Commercially available primers and probe for reference GAPDH gene were used for normalization (Applied Biosystems). The probe was labeled with VIC dye and to avoid competition in the PCR reaction tube, the concentrations of primers were adjusted.

All PCR reactions were performed on the ABI Prism 7700 Sequence Detection System (Applied Biosystems) using the fluorescent Taqman methodology. The PCR cycle at which the fluorescence arises above the background signal is called the Cycle threshold (Ct) and it is inversely proportional to the log of the initial copy number.

In total 10 μl of the reverse transcription volume was used for each PCR reaction in a total volume of 50 μl. A real time PCR reaction was carried out. One primer pair amplified the target gene and the other amplified the endogenous reference gene. Primer and probe concentrations for the target gene were optimized according to the manufacturer’s procedure.

For CEBPα mRNA; the concentrations were 15 pmol for forward and reverse primers and 10 pmol for the probe. The thermal cycling conditions comprised 10 min at 95 °C, 15 s at 95 °C (~45 cycles of 15 s denaturation at 95 °C) and 60 s annealing at 60 °C.

Statistical analysis

Data showed non-parametric distribution & Mann–Whitney U test was used for comparisons between two groups. This test is the non-parametric alternative to Student’s t-test.

Chi-square (χ²) test was used for studying the comparisons and associations between different qualitative variables.

Spearman’s correlation coefficient was used to determine significant correlations between CEBPA expression and different variables.

Kaplan–Meier survival curve was constructed for survival analysis.

The significance level was set at P ≤ 0.05. Statistical analysis was performed with IBM/SPSS Statistics Version 20 for Windows.

Results

Patients characteristics

Characteristics of AML patients are shown in (Table 1).

Results of morphological examination, cytochemical staining and immunophenotyping performed on peripheral blood and bone marrow samples showed that the most common subtype of AML in our study population according to the FAB classification was 19(47.5%) cases of M3. Nine cases (22.5%) of M1, 5 cases (12.5%) of M5b, 2 cases (5%) of M5a, 2 cases (5%) of M0, 2 cases of M2 (5%) and 1 case (2.5%) of M6. No M7 was seen in our study.

Expression of CEBPα mRNA

ΔΔCt method

To quantify the relative expression of CEBPα mRNA, the Ct (threshold cycle) values were normalized for endogenous reference (ΔCt = Ct_sample–CtGAPDH) and compared with a calibrator, using the ‘delta–delta Ct method’ (ΔΔCt = ΔCtSample_Ccontrol). As calibrator we used the average CEBPα mRNA Ct value in the 20 P.B and B.M samples of healthy volunteers.

Using the ΔΔCt value, the relative expression was calculated (2^(-ΔΔCt)) [14].

CEBPα mRNA expression results were classified into two subgroups: Low and High expression according to CEBPα mRNA expression mean level of the healthy population. CEBPα mRNA expression in the healthy population had a mean of 0.97 (considered as cutoff value) using the ΔΔCt method. Low values below the control mean ( < 0.97) was considered “Low” expression. High values above the control mean ( >0.97) was considered “High” expression.

The results obtained using this method showed low expression of CEBPα mRNA in 29 (72.5%) out of the forty patients (values of CEBPα mRNA expression were below the cutoff).

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Patients’ characteristics.</th>
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<tbody>
<tr>
<td>Characteristics</td>
<td>No = 40</td>
</tr>
<tr>
<td>Gender</td>
<td></td>
</tr>
<tr>
<td>Male (no, %)</td>
<td>22 (55%)</td>
</tr>
<tr>
<td>Female</td>
<td>18 (45%)</td>
</tr>
<tr>
<td>Age (years)</td>
<td></td>
</tr>
<tr>
<td>Median (range)</td>
<td>30 (12–77)</td>
</tr>
<tr>
<td>Hb ([Males: 13–18; females: 12–16] g/dl)</td>
<td></td>
</tr>
<tr>
<td>Mean ± SD</td>
<td>7.9 ± 1.88</td>
</tr>
<tr>
<td>WBCs ([4–11] × 10⁹/l)</td>
<td></td>
</tr>
<tr>
<td>Median (range)</td>
<td>20.000(2300–419000)</td>
</tr>
<tr>
<td>Platelets ([150–450] × 10⁹/l)</td>
<td></td>
</tr>
<tr>
<td>Median (range)</td>
<td>56.000(6000–99000)</td>
</tr>
<tr>
<td>P.B. blasts (%)</td>
<td></td>
</tr>
<tr>
<td>Median (range)</td>
<td>23 (4–85)</td>
</tr>
<tr>
<td>B.M. blasts (%)</td>
<td></td>
</tr>
<tr>
<td>Median (range)</td>
<td>45 (20–90)</td>
</tr>
</tbody>
</table>
with median level of 0.19 (range 0–0.87) while the other eleven patients (27.5%) had higher expression of CEBPα mRNA (values were above the cutoff) with a median of 1.52 (range 1.07–2).

Comparison between CEBPα mRNA expressions in relation to FAB subtypes

In cases with all FAB subtypes & M3 subtype; there was a statistically significant difference between cases with low and higher CEBPα mRNA expression ($P$-value = <0.001).

M3 Cases showed statistically significantly higher CEBPα mRNA expression values than other FAB subtypes ($P$-value = 0.036) (Fig. 1).

Cytogenetics and CEBPα mRNA expression in our study group

In our study population, 6 cases (15%) have positive t(8;21), 4 cases (10%) have positive CEP8 and among 19 cases of M3, 15 (78.9%) have positive t(15;17) and 4 (21.1%) were M3 variant (hypogranular) subtype with negative t(15;17).

There was no statistically significant difference between CEP8 and t(8;21) distributions in cases with high and low CEBPα mRNA expression. As regards t(15;17), cases with higher expression showed statistically significantly higher prevalence of positive t(15;17) than cases with low expression (Table 2).

CEBPα mRNA expression and response to therapy

Disease free survival (DFS)

When our patients were stratified according to CEBPA expression subgroups, it was found that the median DFS of all cases was 0.17 years, patients with Low CEBPA expression had a DFS with range of (0.08–4) years and patients with higher CEBPA expression had DFS with range of (0–2) years and this was of no statistical significance ($P$ = 0.95) (Table 3 & Fig. 2).

Overall survival (OS)

The median OS for all cases was 0.33 years. Low CEBPα mRNA expression patients had OS with range of (0.08–4.17) years while patients with Higher CEBPα mRNA expression had OS with range of (0.17–2.17) years with a high statistically significant difference ($P = 0.047$) (Table 4 and Fig. 3).

Discussion

In the last decade, somatically acquired mutations have been identified in several genes in cytogenetically normal (CN) AML: the nucleophosmin 1 (NPM1) gene, the fms-related tyrosine kinase 3 (FLT3) gene, the CCAAT/enhancer binding protein alpha (CEBPA) gene, the myeloid-lymphoid or mixed-lineage leukemia (MLL) gene, the neuroblastoma Ras viral oncogene homolog (NRAS) gene, the Wilms tumor 1 (WT1) gene, and the runt-related transcription factor 1 (RUNX1) gene [15,16]. These gene mutations are most prevalent in CN-AML; however, they also occur in AML with abnormal karyotypes [17].

C/EBPA is a key mediator of normal myeloid differentiation, contributing to both granulopoiesis and monopoiesis. C/EBPA also inhibits cell cycle progression and stimulates cell survival. Alterations in the CEBPA gene or in pathways that down-modulate C/EBPA expression at the transcriptional, translational, or posttranslational levels likely contribute to myeloid transformation by inhibiting myeloid differentiation while favoring myeloid progenitor cell cycle progression [20].

Impairments in C/EBPA signaling such as reduced mRNA or protein expression, aberrant post translational modifications (phosphorylation) and the presence of (dominant-negative) mutations are often observed in human myeloid leukemias which may subsequently contribute to leukemic transformation [13]. Mutations in the transcription factor CCAAT/enhancer binding protein α (CEBPA) are found in 5–14% of the patients with AML [18].

Our study measures only mRNA expression of CEBPA, which may or may not reflect protein expression levels in AML. We found that there was statistically significant difference between CEBPα mRNA expression where the majority of patients 29/40 (72.5%) had low CEBPα expression levels while 11/40 (27.5%) cases showed higher expression levels. This is in accordance with the results previously reported by

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Table 2  Cytogenetics and CEBPα mRNA expression in our study group.

<table>
<thead>
<tr>
<th>FISH analysis</th>
<th>Low expression</th>
<th>Higher expression</th>
<th>$P$ value</th>
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<tbody>
<tr>
<td>t(8;21) (No. = 40)</td>
<td>Positive</td>
<td>4(13.8%)</td>
<td>2(18.2%)</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>25(86.2%)</td>
<td>9(81.8%)</td>
</tr>
<tr>
<td>t(15;17) (No. = 40)</td>
<td>Positive</td>
<td>8(27.6%)</td>
<td>7(63.6%)</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>21(72.4%)</td>
<td>4(36.4%)</td>
</tr>
<tr>
<td>Cep 8 (No. = 40)</td>
<td>Positive</td>
<td>3(10.3%)</td>
<td>1(9.1%)</td>
</tr>
<tr>
<td></td>
<td>Negative</td>
<td>26(89.7%)</td>
<td>10(90.9%)</td>
</tr>
</tbody>
</table>

Cep 8, centromere enumeration probe 8.

* Significant at $P \leq 0.05$.
Barjesteh et al. (2003) and D’Alò et al. (2008) reported that the majority of their patients showed low CEBPA expression level [14,19].

The CEBPA gene is down-regulated in APL and its upstream promoter region is highly methylated. However, the mechanisms regulating CEBPA expression in APL have not been completely identified. The data reported by Guibal et al. [22], support a role for CEBPA deregulation in APL genesis. In our work, we observed that M3 cases showed statistically significantly higher CEBPA expression values than other FAB subtypes. This is in agreement with the previously reported data from Santana-Lemos et al. (2011) who observed that patients with acute promyelocytic leukemia (M3) presented with higher levels of CEBPA expression than other acute myeloid leukemia subtypes [22] and Pabst et al., who reported that acute promyelocytic subtype (M3) of AML is characterized by the presence of the PML–RARA fusion protein the product of t(15;17) and over expression of C/EBPA [13].

Our 40 patients received induction therapy and were included in the survival analysis. Clinical outcome after a median duration follow up of 6 months was investigated based on CEBPA expression levels. Higher C/EBPA expressing patients had better survival rates, while low expressing patients had poor survival rates.

Conclusion and future directions

Using Real Time PCR, we were capable to define two main subgroups of patients with low and high CEBPA m-RNA expression, although we observed heterogeneous CEBPA expression among different FAB subtypes. Regarding the clinical outcome, higher CEBPA expressing patients had better survival rates, while low expressing patients had poor survival rates.

Restoration of C/EBPA expression in AML might provide a means to induce cell differentiation and slow cell proliferation to contribute to AML therapy [20]. The potential for differentiating therapy to improve cure rates in AML is exemplified by the use of ATRA for the treatment of acute promyelocytic leukemia (APL). High doses of ATRA have been shown to dissociate the PML–RARA fusion product associated with RARA-responsive genes, thereby converting PML–RARA to an activator allowing differentiation to ensue.

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References


