C/EBPα Expression in Egyptian patients with Acute Myeloid Leukemia

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Abstract

Purpose: The CCAAT/enhancer binding protein alpha (C/EBPα or CEBPA) transcription factor plays an important role in myeloid differentiation. Alterations in C/EBPα levels have been described in AML.
Patients and methods: We studied the C/EBPα expression level by real-time PCR in 49 newly diagnosed AML patients.
Results: Low C/EBPα expression was observed in 40/49 (81.7%, median 0.018) compared to normal subjects (median 1.26) (p = 0.001). We observed that low C/EBPα expression was associated with low hemoglobin level (p = 0.001), high HLA-DR expression (p=0.013) and poor disease free survival (DFS) and overall survival (OS).
Conclusion: low C/EBPα expression defined a subgroup of AML patients with poor DFS and OS, so can be used with other markers to assess prognosis of AML patients.

Introduction

Acute myeloid leukemias (AML) are clonal disorders that are characterized by block in differentiation along one or more hematopoietic lineages. Molecular abnormalities are frequently detected in AML. The genetic alterations in AML often affect transcription factors that also have an important role in normal haematopoiesis [1].

The CCAAT/enhancer binding protein alpha (C/EBPα or CEBPA) transcription factor regulates the balance between cell proliferation and differentiation in haematopoietic and non haematopoietic tissues [2].

The main role of C/EBPα in haematopoiesis is in the development of granulocytes [3]. A critical role for the function of C/EBPα in granulopoiesis was demonstrated in mice harbouring a disruption of the C/EBPα gene [4]. These mice showed a selective early block in granulopoiesis, with the appearance of many myeloid blasts in foetal liver and peripheral blood [5].

C/EBPα affects haematopoietic cell fate decisions by inducing myeloid differentiation and inhibiting erythroid differentiation in progenitors more primitive than granulocyte/monocyte progenitors (GMPs) [3]. C/EBPα also plays a regulatory role in maintenance of the hematopoietic stem cell (HSC) population, since both C/EBPα-deficient fetal liver cells and adult bone marrow cells display a competitive advantage over wild-type bone marrow cells in transplantation experiments [6].

C/EBPα is a 42-kDa transcription factor that possesses a DNA-binding basic region/leucine zipper domain (bZIP) in the COOH terminus and two transactivation domains TAD 1 and TAD 2 in the NH2 terminus [7]. C/EBPα dimerizes via its leucine zipper domain and then binds DNA via the adjacent basic region. Once bound to DNA, C/EBPα mediates transactivation via its NH2-terminal [8]. C/EBPα mutations have been observed in AML patients with the approximate frequency of 5–14% [9]. The mutations can be largely divided into two common types. First, carboxy- terminal in-frame mutations disrupt the basic zipper region, thus affecting DNA binding as well as homo and heterodimerization with other C/EBPα family members. These mutations are in most AML cases in one of the two C/EBPα alleles and are often associated with a second mutation in the other allele, which usually leads to loss of C/EBPα function. Second, amino terminal frame shift mutations result in premature termination of the normal 42 kDa form of the C/EBPα protein while preserving the 30 kDa form leading to the induction of proliferation [6].

The block in myeloid differentiation, combined with an enhanced self-renewal, closely resembles some characteristics of leukemic development. Indeed, C/EBPα has been suggested to be a common denominator in human acute myeloid leukemia (AML) [10].

In the present study, real-time PCR was performed to assess the expression of CEBPα in AML patients and its association with clinical and hematological data, FAB subtypes and immunophenotyping.

Subjects and Methods

This study included 49 newly diagnosed AML patients attending at Kasr El-Aini Center of Oncology and Radiation NEMROCK, faculty of medicine, Cairo University. They were diagnosed according to morphology and immunophenotype and classified according to FAB criteria. The patients group included 30 females and 19 males with a ratio of 1.6:1, their age ranged between 2 and 77 years (median 30 years). Twenty control subjects attending for other health problems were also included in this study.

One ml bone marrow samples were obtained from AML patients in sterile EDTA vacutainers. One ml EDTA bone marrow sample from 3 control subjects attending for bone marrow aspirate for splenectomy and 2 ml peripheral blood samples were obtained from 17 control subjects. Informed consent was obtained from all patients and controls. FISH analysis for t(15;17), t(8;21) and trisomy 8 was available for 41 patients. The mononuclear cells were separated and total cellular RNA was extracted from the mononuclear cells using the QIA amp RNA blood Mini kit (QIAGEN, Catalogue number. 52304), followed by cDNA preparation using a high capacity cDNA archive kit (Applied Biosystem, Foster city, CA, USA).

Detection of C/EBPA expression by quantitative real-time PCR:

Real-time PCR was performed by ABI prism (7700) TaqMan instrument (ABI Prism 7700, Applied Biosystem, USA) according to the method done by ***Barjesteh et al., 2003*** [11] using 25 ul mix containing 2 µl cDNA, 12.5 µl master mix, 1 ul of each primer (15 pmol), 0.5 µl probe (10 pmol), 1.25 µl GAPDH and 6.75 nuclease free water. Two µl water instead of cDNA was used as control for any contamination. The primers used for C/EBPA were F: 5'-TCGGTGGACAAGAACAG-3' and R: 5'- GCAGGCGGTCATT -3' (Operon, Germany). The used probe is [6-FAM]-ACAAGGCCAAGCAGCGC-[TAMRA-6-_FAM] (Operon, Germany). The thermal cycler conditions were 10 minutes at 95˚C followed by 45 cycles of denaturation for 15 seconds at 95˚C and, annealing /extension at 60˚C for 30 seconds.

To determine the expression levels of the C/EBPα in unknown samples, the Ct (cycle threshold) were normalized for endogenous reference ($\Delta Ct = Ct_{target} - Ct_{GAPDH}$) and compared with a calibrator, using the ΔΔCt method [$\Delta\Delta Ct = (Ct_{C\backslash EBP\alpha} - Ct_{calibrator})$]. As a calibrator, the C\EBPα levels in the 20 control subjected were used. Then $2^{-\Delta\Delta Ct}$ was used for calculation.

Statistical analysis

Data was coded and entered using the statistical package (SPSS) version 15. Data was summarized using median and range for quantitative variables while number and percent were used for qualitative variables. Comparison between groups were done using chi square test for qualitative variables while analysis of variants (ANOVA) with multiple comparisons (post Hoc test) was used for normally distributed quantitative variables. Non parametrical Kruskal-Wallis test and Mann-Whitney test were used for quantitative variables not normally distributed. Correlations were done to test for linear relation between quantitative variables. Kaplan-Mayer test was done to show the survival of AML patients and Log-Rank test was done to compare the survival time among C/EBPα subgroups. P value ≤ 0.05 was considered statistically significant.

Results

This study analyzed the C/EBPα expression among 49 newly diagnosed AML patients of different FAB subtypes and 20 control subjects by real-time PCR technique. There was difference in C/EBPα expression between PB and BM mononuclear cells among control subjects. The clinical and laboratory characteristics of studied AML patients are shown in table-1.

C/EBPα expression was significantly lower in AML patients (median 0.022) in comparison to the control subjects (median 1.26), p = 0.001.

An expression level in the range of 95% confidence interval (CI) (0.72–2.11) defined for the control subjects was considered as intermediate expression. Values below the lower boundary of the 95% CI (< 0.72) were considered low expression while values above the upper boundary of the 95% CI (> 2.11) were considered high expression. This was done according to ***Barjesteh et al., 2003*** [11]. Forty of our 49 AML patients (81.7%) showed low expression (median 0.018,), 6/49 (12.2%) showed intermediate expression (median 1.5) while 3/49 (6.1%) showed high expression (median 4.92).

C/EBPα expression levels in AML (Table 2):

C/EBPα expression according to age and sex:

No statistical significant difference was observed as regards the age and sex when comparing the three C/EBPα expression groups.

C/EBPα expression and laboratory characteristics:

A statistical significant difference was observed between low expression and high expression groups as regards the Hb level (p=0.002) while no significant differences were found regarding the WBCs count, platelet count, P.B and BM blast cells percentage.

Positive correlation was found between Hb level and C/EBPα percentage expression (p=0.001). On the other hand, negative correlation was found between HLA-DR and C/EBPα percentage expression (p=0.013). No correlation was found between C/EBPα expression and other hematological findings.

C/EBPα expression and FAB subtypes:

Different C/EBPα expression levels were observed in different FAB subtypes but not reaching statistical significant difference. The cases with high expression belonged to M1 and M3 FAB subtypes (Fig. 1).

C/EBPα expression as regards the immunophenotyping and cytogenetic studies:

Regarding immunophenotyping, the low expression group showed the highest HLA-DR percentage expression with a statistical significant difference observed between low expression and high expression group (p = 0.037) but, conversely with no statistical significant difference regarding CD34 and CD33 (p=0.149 and p =0.127, respectively).

FISH analysis was done for only 41 patients. One third of the patients (9 cases) with low C/EBPα expression were lying in the favorable cytogenetic risk group but again with no statistical significant difference among cases with low and high expression.

C/EBPα expression and disease outcome:

The median follow up duration for patients was 18 months. The disease free survival and overall survival in the 49 patients were 33.3% (Median 9 months) and 20.4% (Median 4 Months) respectively. A significant difference was found in the DFS and OS between low expression group and intermediate and high expression groups (p = 0.001 and p < 0.001 respectively), (Fig. 2).

Discussion

Several human tumor types display reduction in the level of C/EBPα, suggesting that C/EBPα is a tumor suppressor [12]. However, this character has been only obtained in myeloid leukemias [13]. C/EBPα promotes differentiation by the upregulation of lineage specific gene products and by the exit of cell cycle that means proliferation arrest [13].

According to the previous data, we found most of AML patients (81.6%) involved in our study showing low C/EBPα expression while only 18.4% of patients showed intermediate and high expression. On the other hand ***Barjesteh***

et al., 2003 [11] reported that 50.9% (141/277) showed intermediate expression and 46.5% (127/277) showed high expression and only 3.2% (9/277) showed low expression. Also ***D'Alò et al., 2008*** [14] found that C/EBPα expression of his AML patients was similar to that of normal bone marrow mononuclear cells.

Low C/EBPα expression patients showed significant low hemoglobin in comparison to high expression patients while ***Barjesteh et al., 2003*** [11] found that low C/EBPα expression was associated with low WBCs and platelet count, ***D'Alò et al., 2008*** [14] observed low level of C/EBPα expression in AML cases with leucopenia (WBCs < 4 x 10^9/L).

No correlation was observed between C/EBPα level and bone marrow blasts, this is in agreement with ***D'Alò et al., 2008*** [14].

The nonlineage specific marker HLA-DR was significantly expressed on blasts of low C/EBPα expression patients in comparison to high C/EBPα expression patients who showed low HLA-DR expression, and ***D'Alò et al., 2008*** [14] reported that C/EBPα expression was associated with the more mature blast phenotype positive for CD33 and CD11c. This may be explained by what is reported by ***Schuster and Porse, 2006*** [13], that C/EBPα promotes differentiation by the upregulation of lineage specific gene products.

Most of our patients with different FAB subtypes showed low expression with no statistical significant difference among them probably due to the low compare our results with ***D'Alò et al., 2008*** [14] who reported low C/EBPα expression in acute erythroid leukemia.

Heterogeneous results regarding cytogenetic subgroups were observed. We observed that 1/3 of the patients with low C/EBPα expression were lying in the favorable risk group. This is in agreement with ***Barjesteh et al., 2003*** [11] and can be explained by ***Pabst et al., 2001***[15] who reported that t(8;21) gives rise to a fusion gene encoding AML1-ETO protein which appears to suppress C/EBPα expression directly by inhibiting positive autoregulation of the C/EBPα promoters. Moreover, the application of AML1-ETO siRNA followed by stimulation with inducers of differentiation caused high expression of C/EBPα in comparison to these inducers alone.

In our study a statistical significant association was found between C/EBPα expression and disease outcome, as patients with low C/EBPα expression were associated with poor DFS and OS (significant) as compared to patients with intermediate and high C/EBPα expression. This is in agreement with ***Barjesteh et al., 2003*** [11] who reported that low C/EBPα expression seemed to have a relatively poor OS and EFS (not significant).

In conclusion, we studied C/EBPα expression in AML patients using real-time PCR. We found that low C/EBPα was associated with low hemoglobin concentration, high HLA-DR expression and poor DFS and OS. Thus, we conclude that low C/EBPα expression defined a subgroup of AML patients with poor DFS and OS, so can be used with other markers to assess prognosis of AML patients.

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Tables

Table 1: Clinical and laboratory characteristics of AML patients

Character	No.
Sex	
Male	19
Female	30
Age	
Median (range)	30 (2-77)
Laboratory data	
WBC count (x10x3/ul)	
Median (range)	15 (2.3-90)
Hb (g/dl)	
Median (range)	7 (4.7-12)
Platelet count (x10x3/ul)	
Median (range)	41 (10-198)
P.B blasts (%)	
Median (range)	30 (0-97)
B.M blasts (%)	
Median (range)	80 (34-100)
FAB subtypes	
M0	1
M1	20
M2	4
M3	10
M4	3
M5	8
M7	1
Bilineage	2
Immunophenotyping	
CD34 (%)	59.1 (12.9-95.4)
Median (range)	
CD33 (%)	81.7 (37.1-99.2)
Median (range)	
HLA-DR (%)	69.15 (3.2-92.2)
Median (range)	
Cytogenetic subgroups	
Favorable [t(8;21) and t(15;17)]	13
Unfavorable (trisomy 8)	1
Others	27

Table 2: Clinical and laboratory characteristics of AML patients according to C/EBPα expression

Character	Low (<0.72)	Intermediate (0.72-2.11)	High (>0.2.11)
Sex			
Male	14	4	1
Female	26	2	2
Age			
Median (range)	30 (2-77)	30.5 (22-52)	33 (14-45)
Laboratory data			
WBC count ($x10^{x3}$/ul)			
Median (range)	16.5 (2.3-86.3)	16.9 (7.2-90)	11.5 (2.8-44.5)
Hb (g/dl)			
Median (range)	6.8 (4.7-10.9)	8.4 (5.4-10.6)	11 (9.3-12)
Platelet count ($x10^{x3}$/ul)			
Median (range)	39.5 (10-198)	50.5 (22-84)	72 (28-93)
P.B blasts (%)			
Median (range)	30 (0-80)	27 (10-54)	26 (0-35)
B.M blasts (%)			
Median (range)	85 (34-100)	85 (66-97)	77 (36-92)
FAB subtypes			
M0	1	-	-
M1	17	2	1
M2	3	1	-
M3	7	1	2
M4	3	-	-
M5	6	2	-
M7	1	-	-
Bilineage	2	-	-
Immunophenotyping			
CD34 (%)			
Median (range)	60.3 (12.9-95.4)	37.1 (29.9-65.3)	76.5 (42.3-81.3)
CD33 (%)			
Median (range)	80.5 (37.1-99.2)	94.5 (77.3-98.8)	84.5 (70.4-95.5)
HLA-DR (%)			
Median (range)	70.1 (10.6-92.2)	37.6 (5-98.8)	14.9 (3.2-28.9)
Cytogenetic studies			
Favorable	9	2	2
Unfavorable	-	1	-
Others	21	4	2

Figures

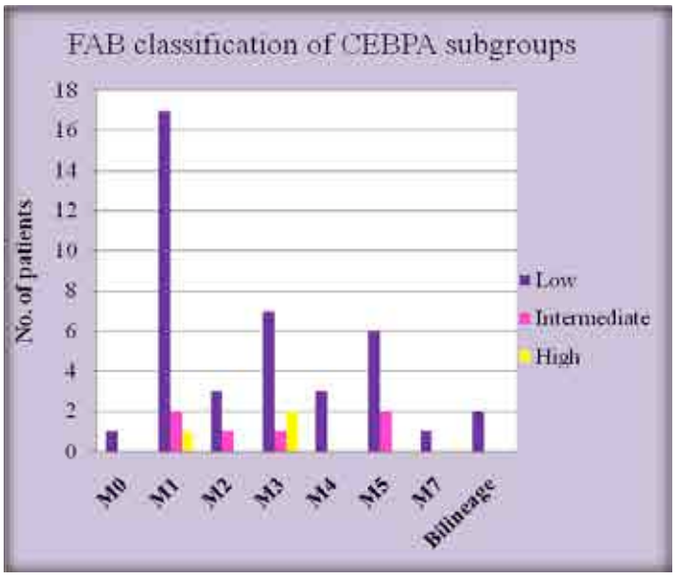

Fig. 1: C/EBPα subgroups and FAB subtypes in our study group.

a b

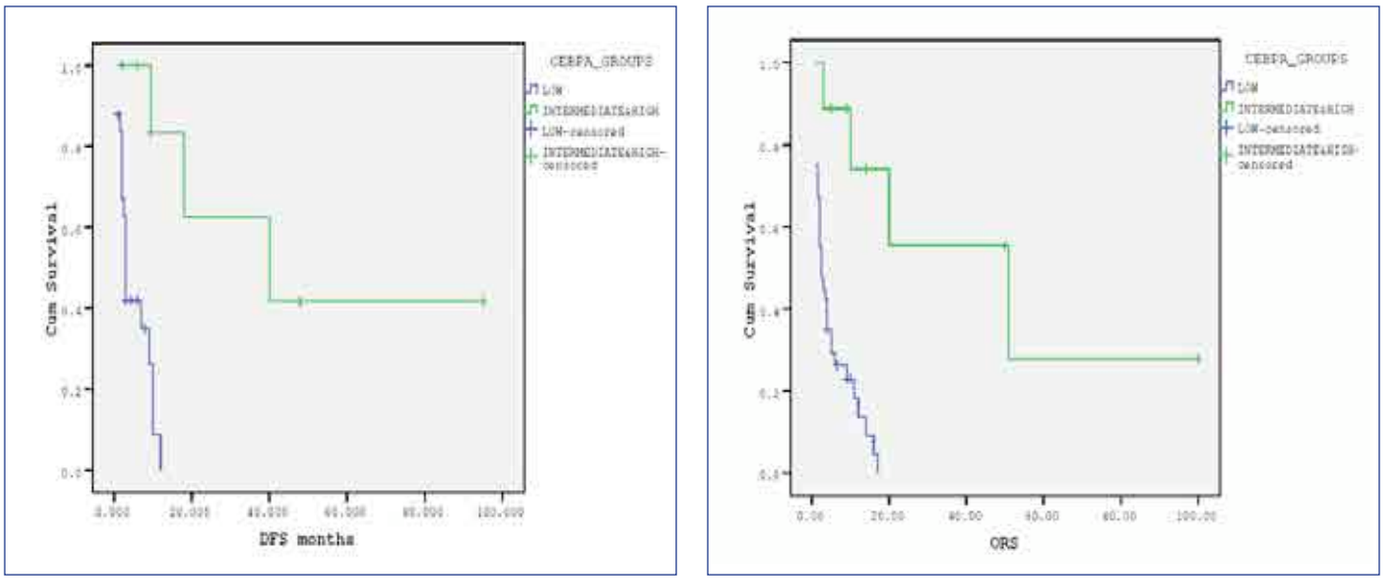

Fig. 2: Disease free survival (a) and overall survival (b) of AML patients based on C/EBPα expression levels.