Prevalence and Prognostic Impact of CEBPA Gene Mutation (Simplified Assay Technique) in Egyptian Acute Myeloid Leukemia Patients with Normal Cytogenetics

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Received: 23 July 2015 / Accepted: 26 November 2015 / Published online: 30 November 2015
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Abstract Mutations of the CCAAT/enhancer binding protein alpha (*CEBPA*) gene have been associated with a favorable outcome in patients with acute myeloid leukemia (AML), especially in those with a normal cytogenetics. However, few studies were done on Egyptian AML patients and none of them look for easier and less expensive method for *CEBPA* mutation screening. This study is aimed to investigate the prevalence of *CEBPA* mutations and its clinical and prognostic impact in Egyptian patients with cytogenetically normal AML (CN-AML). This was done using fragment analysis to assess this method as a cheaper and less laborious screening method compared to sequencing. Fluorescent PCR was done to amplify *CEBPA* gene in DNA extracted from 40 CN-AML patients. This was followed by fragment analysis of post-PCR products using GeneMapper software for detection of *CEBPA* mutations. *CEBPA* gene mutations were found in 7/40 CN-AML patients (17.5 %) and it was significantly associated with lower LDH levels ($p = 0.039$). All patients with *CEBPA* mutations achieved clinical remission and none of them showed refractoriness, relapsed, or died by the end of the 2 years study period. Furthermore, those patients demonstrate significantly longer overall and disease free survival than those with wild type *CEBPA* gene

($p = 0.001$ and 0.004 respectively). *CEBPA* mutation has a favorable prognostic impact in CN-AML. Fragment analysis is a good, less laborious and cheaper method that can be used for *CEBPA* mutation screening in patients with CN-AML.

Keywords AML · *CEBPA* · Fragment analysis

Introduction

Acute myeloid leukemia (AML) is a heterogeneous group of hematopoietic stem cell disorders characterized by the expansion of undifferentiated myeloid progenitors [1]. Currently, cytogenetic findings provide the most important prognostic information and are used to guide risk-adapted treatment strategies [2]. Cytogenetically normal AML (CN-AML) represents 40–50 % of all AML cases and currently is considered separate entity in WHO classification [3]. The identification of molecular markers that precisely differentiate a patient's risk could improve treatment outcome and deeply refine the prognosis of patients with CN-AML [4, 5].

CCAAT/enhancer binding protein alpha (*CEBPA*) is a transcription factor encoded by *CEBPA* gene that is located at chromosome 19q13. It was originally isolated from rat liver that bound to viral enhancer sequences CCAAT [1, 4, 5]. *CEBPA* is the original member of the basic region leucine zipper (bZIP) class of transcription factors [6]. *CEBPA* protein consists of two N-terminal transactivation domains, a basic DNA binding region, and a C-terminal leucine zipper domain [7]. Wild-type (wt) *CEBPA* exists in two translational isoforms, p42 and p30 [8].

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CEBPA is expressed in myeloid precursors during hematopoiesis, where it co-ordinates cellular differentiation and regulates the expression of several granulocyte-specific genes [6]. Therefore, it is important for commitment to the granulocytic lineage and differentiation of mature neutrophils [7–9].

CEBPA mutations have been reported in approximately 5–14 % of all AML with predominance in CN-AML [5, 10, 11]. It has been reported that suppressed *CEBPA* function characterizes CN-AML with favorable outcome [11, 12]. Accurate testing for *CEBPA* mutations is important for identification of this prognostically significant AML subtype which is now considered as a distinct disease entity in the 2008 World Health Organization classification of leukemia's [5, 7].

The aim of the present study is to assess the prevalence of mutations in the *CEBPA* gene in Egyptian CN-AML patients using fragment analysis and to evaluate its relation to the patient clinical findings and outcome.

Materials and Methods

Patients and Samples

This study was carried out on 40 CN-AML patients that include 10 males and 30 females with age ranging from 25 to 71 years (mean $\pm$ SD; 44.4 ± 13.7). The patients were followed up for 24 months (if still alive) or until death incident. The duration of follow up ranged from 1 to 24 months (median 6 months). The diagnosis of AML was based on clinical findings, morphological examination and flow cytometric immunophenotyping of their peripheral blood (PB) and bone marrow (BM) samples. All samples were collected at time of initial diagnosis. To establish CN-AML, pretreatment samples from all patients were studied by G–R banding analysis, more than 20 metaphase cells from diagnostic BM had to be analyzed and reported as normal karyotype according to the international system for human cytogenetic nomenclatures (ISCN) [13]. In addition, PB samples were collected from 10 healthy adults of matched sex and age to confirm the median fragment sizes of normal *CEBPA* gene PCR products sizes.

Methods

DNA Extraction and Polymerase Chain Reaction

Genomic DNA was extracted from the diagnostic PB/BM samples Using QIAamp DNA blood mini kit (provided by QIAGEN cat. No. 51104, USA) according to the manufacturer's protocol. To amplify *CEBPA* gene, 3 pairs of

primers were used; *TAD1F* (HEX): *5'TCGGCCGACTTCTACGAGGC3'*, *TAD1R*: *5'GCGCCCGGGTAGTCAAAGT3'*, *TAD2F* (NED): *5'TACCTGGACGGCAGGCTGGA3'*, *TAD2R*: *5'TGCAGGTGCATGGTGGTCT3'*, *bZIPF* (FAM): *5'AGAAGTCGGTGGACAAGAACA3'*, *bZIPR*: *5'AGTTGCCCATGGCCTTGAC3'*, that cover the entire coding region of human *CEBPA* gene were used [14]. The forward primers in each pair were fluorescently labeled to enable post PCR fragment analysis using Gene Mapper analysis (Applied Biosystems, Foster City, CA, USA).

PCR reactions were performed in a total volume of 25 μ L containing 12.5 μ L of Maxima hot start PCR master mix 2X (Thermo Scientific), 0.1 μ L of each primer (100 pmol) (Applied Biosystems), 5 μ L of Q-solution (Applied Biosystems), 1 μ L of extracted DNA and the reaction was completed to 25 μ L using 5.9 μ L of molecular grade water. Thermal cycling was performed using a 9700 thermocycler (PE Applied Biosystems, USA) with preactivation at 95° C for 4 min then 30 cycles of denaturation at 94° C for 30, annealing at 55° C for 90 s, and extension at 72° C for 1 min. This is followed by post extension at 72° C for 10 min.

Fragment Analysis of Post-PCR Products Using GeneMapper Software

Fragment analysis was done for the fluorescent PCR product of our samples to detect the mutant forms of *CEBPA* gene that has different fragment length. This was done on ABI 310 Genetic analyzer (PE Applied Biosystems, USA) using Gene Mapper software (PE Applied Biosystems, USA). 1 μ L of PCR product was added to 10 μ L Hidiformamide (Applied Biosystems) and 1 μ L of GeneScan 600 LIZ internal size standard (Applied Biosystems) and introduced into the ABI310 genetic analyzer.

To establish cutoff values for the fragment size, of *CEBPA* gene was amplified in 10 PB samples from normal control group and the average fragment size for the three PCR products were calculated. If the fragment sizes fell outside $+1$ or -1 of this average, it is considered mutant (bZIP: 239.4–240.3 bp, TAD1: 314.8–316.2 bp, TAD2: 244.7–245.5 bp).

Statistical Analysis

The results were expressed as the mean $\pm$ SD or percentage. Statistical analysis was carried out using the SPSS Statistics 17.0 program. The Chi square test and independent samples and *T* test was used to compare the frequencies of various characteristics between patients with mutant and wild type *CEBPA*. A value of $p < 0.05$ was considered statistically significant.

Results

Prevalence of CEBPA Mutations

Among the 40 patients with CN-AML, 7 patients showed mutant *CEBPA* (17.5 %). b-ZIP, TAD-1 and TAD-2 occur in 3, 1, and 3 patients respectively (7.5, 2.5, and 7.5 % respectively). Figure 1 demonstrates examples of GeneMapper results for *CEBPA* gene amplicons.

CEBPA Mutation and Clinical Parameters

There was no significant difference between *CEBPA*-mut and *CEBPA*-wt patient groups in the demographics, clinical findings, or laboratory results except for LDH which was significantly lower in the mutant patient group ($p = 0.039$).

Prognostic Impact of CEBPA Mutations

Of the total 40 patients, 20 (50 %) achieved CR. All patients in the *CEBPA*-mut group (7/7, 100.0 %) achieve

CR in contrast to only 13/33 (39.4 %) of *CEBPA*-wt patients ($p = 0.014$) (Table 1). In addition, none of *CEBPA*-mut patients who achieved CR relapsed while 7/13 (53.8 %) of *CEBPA*-wt patients relapsed ($p = 0.044$). Furthermore, none of the *CEBPA*-mut patients were refractory to treatment and none of them died by the end of our study compared to 7 refractory (21.2 %, $p < 0.0001$) and 27 died patients in the *CEBPA*-wt group (81.8 %, $p < 0.0001$) (Table 1).

Survival Analysis

At the end of our 2 years study, all 7 mutant patients were still alive while only 6/33 *CEBPA*-wt patients (18.8 %) remains alive ($p < 0.0001$). The median duration of overall survival (OS) for *CEBPA*-mut group was 24 months compared with 5 months for the *CEBPA*-wt group ($p = 0.001$). Similarly, the median duration for the disease free survival (DFS) of the *CEBPA*-mut group was significantly longer than that for the *CEBPA*-wt group (23 vs. 16 month, $p = 0.004$) (Table 1 and Fig. 2).

Fig. 1 Examples of genemapper electropherogram from fluorescent multiplex PCR of *CEBPA* gene (bZIP; upper, TAD1; middle, and TAD2; lower) demonstrate wild and mutated fragments

Table 1 Patients' characteristics according to the *CEBPA* mutational status

Character	Total (n = 40)	Mutant (n = 7, 17.5 %)	Wild (n = 33, 82.5 %)	p
Age (mean $\pm$ SD)	44.4 $\pm$ 13.7	39.8 $\pm$ 11.8	45.4 $\pm$ 14.1	0.929
Gender (M/F)	10/30	2/5	8/25	0.572
<i>Clinical characteristics no (%)</i>				
Bleeding tendency	28 (70.0 %)	6/7 (85.7 %)	22/33 (66.7 %)	0.306
Splenomegaly	16 (40.0 %)	2/7 (28.6 %)	14/33 (42.4 %)	0.408
Hepatomegaly	18 (45.0 %)	2/7 (28.6 %)	16/33 (48.5 %)	0.297
Lymphadenopathy	12 (30.0 %)	2/7 (28.6 %)	10/33 (30.3 %)	0.654
Fever	24 (60.0 %)	5/7 (71.4 %)	19/33 (57.6 %)	0.408
<i>Chemical laboratory findings (mean $\pm$ SD)</i>				
SGPT (U/L)	47.8 $\pm$ 11.6	35.3 $\pm$ 5.7	50.5 $\pm$ 14.0	0.624
SGOT (U/L)	40.2 $\pm$ 6.4	33.2 $\pm$ 7.5	41.7 $\pm$ 7.6	0.620
Albumin (g/dL)	3.8 $\pm$ 0.4	3.7 $\pm$ 0.5	3.8 $\pm$ 0.5	0.787
Bilirubin (mg/dL)	0.75 $\pm$ 0.43	0.7 $\pm$ 0.3	0.8 $\pm$ 0.5	0.516
Creatinine (mg/dL)	0.8 $\pm$ 0.2	0.84 $\pm$ 0.21	0.83 $\pm$ 0.20	0.940
Uric acid (mg/dL)	4.0 $\pm$ 1.8	3.8 $\pm$ 1.1	4.0 $\pm$ 2.0	0.854
LDH (U/L)	695.4 $\pm$ 227.2	536.14 $\pm$ 74.5	729.4 $\pm$ 234.9	0.039
<i>Hematological and morphological findings (mean $\pm$ SD)</i>				
WBCs ($\times 10^3/\mu\text{L}$)	32.9 $\pm$ 30.3	28.0 $\pm$ 23.7	32.9 $\pm$ 30.3	0.693
Hb (gm/dL)	8.7 $\pm$ 2.6	8.8 $\pm$ 2.1	8.7 $\pm$ 2.6	0.908
Platelets ($\times 10^3/\mu\text{L}$)	36.0 $\pm$ 32.0	43.9 $\pm$ 19.2	36.6 $\pm$ 5.6	0.628
BM blasts (%)	68.4 $\pm$ 21.1	70.4 $\pm$ 25.2	68.0 $\pm$ 20.6	0.781
ANC	4.1 $\pm$ 0.8	6.1 $\pm$ 3.3	3.7 $\pm$ 0.7	0.270
<i>FAB classification no (%)</i>				
M1	6 (15.0 %)	1 (14.3 %)	5 (15.2 %)	0.954
M2	12 (30.0 %)	3 (42.8 %)	9 (27.3 %)	0.414
M4	12 (30.0 %)	2 (28.6 %)	10 (30.3 %)	0.928
M5	8 (20.0 %)	1 (14.3 %)	7 (21.2 %)	0.677
M7	2 (5.0 %)	0 (0.0 %)	2 (6.0 %)	0.504
<i>Outcome measures no (%)</i>				
Complete remission	20 (50 %)	7 (100.0 %)	13 (39.4 %)	0.014
Relapse	7/20* (35.0 %)	0 (0.0 %)	7/13* (53.8 %)	0.044
Refractory	7 (17.5 %)	0 (0.0 %)	7 (21.2 %)	<0.0001
Mortality	7 (17.5 %)	0 (0.0 %)	27 (81.8 %)	<0.0001
<i>Survival analysis in studied cases</i>				
OS (month)	13/40 (32.5 %)	7/7 (100.0 %)	6/33 (18.8 %)	0.001 [#]
DFS (month)	13/20 (65 %)	7/7 (100.0 %)	6/13 (46.2 %)	0.004 [#]

* Relapse is calculated out of the cases who achieve CR

[#] Significance Log rank

Discussion

The growth inhibitory and differentiation promoting function of *CEBPA* make it an attractive candidate for tumor suppression. In the last few years, various mechanisms have been suggested through which *CEBPA* is negatively regulated in AML [15]. In our study, we evaluated 40 Egyptian CN-AML patients for the prevalence of different mutations in the *CEBPA* gene, its relation to clinical and laboratory findings, and its prognostic impact. For this purpose, we used a relatively simple test for the detection

CEBPA mutations that involve fluorescent PCR amplification of three *CEBPA* gene targets (b-ZIP, TAD1, and TAD2) followed by product fragment analysis using capillary electrophoresis. *CEBPA* mutations are identified by the appearance of an additional fragment peak of different length relative to the wild-type fragment.

In our study, *CEBPA* mutation was detected in 7 cases out of 40 CN-AML patients (17.5 %). All the detected mutations were single mutations (3 in each b-ZIP, and TAD-2 regions and 1 in TAD-1 region). In previous studies, the incidence of *CEBPA* mutation in AML varies

Fig. 2 Kaplan–Meier curve for disease free survival (DFS) (a) & overall survival (OS) (b) in CEBPA-mut and CEBPA-wtCN-AML patients. A very high significant difference in DFS & OS between CEBPA-mut and CEBPA-wt cases is observed ($p = 0.004$ and 0.001 respectively)

from 11 to 20 % with the CN-AML category having a higher incidence [10, 16, 17]. The absence of double *CEBPA* mutation within our patients may be partly due to low sample number or the limited ability of fragment analysis assay to detect point mutations that will not lead to change in the PCR product fragment length. However, the concordance of *CEBPA*-mut prevalence in our study to other reports in which sequencing was used to detect this mutation indicate that fragment analysis may miss only few cases and it may be used as an alternative, easier, faster and more economic method for screening of AML patients for this mutation.

Although *CEBPA* mutation was observed with lower median age in our study, this difference was not statistically significant. Similarly there was non-significant difference in gender distribution between both groups. This finding is in accordance with previous studies [17, 18]. Though, Green et al. [19] found that younger age was associated with a greater number of mutations.

Among laboratory results, only LDH level showed significant difference between both groups. It was significantly lower in *CEBPA*-mut group than in *CEBPA*-wt group (means 536.1 and 729.4 respectively, $p = 0.039$). This result agreed with some previous reports [17, 18, 20]. However, Frohling et al. [21] reported no significant difference in LDH level between both groups. As regards other laboratory findings, significant lower platelets count and higher blast cells percentage are reported by others [22, 23], but this was not found in our study. This discrepancy in results may be related to the relatively limited number of patients included in the present study.

Regarding FAB classification, M2 was the most frequent FAB subtype in *CEBPA*-mutgroup (3/7, 42.8 %) followed by M4 (2/7, 28.6 %). However, there was no significant difference in FAB classification between *CEBPA* mutated

and wild groups. The prevalence of M2 in *CEBPA* mutated AML was agreed by most of the previous studies [14, 23, 24, 25]. However, others reported M1 as the mostly occurring FAB subtypes in *CEBPA* mutated AML [17, 26, 27].

Another important aspect of *CEBPA* mutation is the association with the clinical outcome. In our study, all *CEBPA*-mut patients achieved CR (7/7, 100.0 %) in contrast to *CEBPA*-wt group in which CR was achieved only in 13/33 (39.4 %) ($p = 0.014$). Within the patients who achieve CR, none in the *CEBPA*-mut group relapsed compared to 53.8 % in *CEBPA*-wt group ($p = 0.044$). Additionally, there was no refractory or died cases (during the duration of the study) in the *CEBPA*-mut group while 7/33 (21.2 %) were refractory to treatment protocol used and 27/33 (81.8 %) died in the *CEBPA*-wt group ($p < 0.0001$ for both). Most of the previous studies showed non-significant difference in the CR between *CEBPA*-mut and *CEBPA*-wt AML patients [17, 21, 26, 27].

At the end of our 2 years study, all 7 mutant patients were still alive (100 %), while only 6/33 *CEBPA*-wt patients (18.8 %) remains alive. Both DFS and OS were 100 % in the *CEBPA*-mut patient group compared to 46.2 and 18.8 % respectively in the *CEBPA*-wt group. The median duration of DFS and OS for the *CEBPA*-mut group (23 and 24 months respectively) was significantly longer than that for the *CEBPA*-wt group (16 and 5 month respectively) with p value 0.004 and 0.001 respectively. Diverse results were obtained from previous studies as regards DFS and OS in *CEBPA*-mut AML patients. Most of these studies demonstrate a significant increase in both OS and DFS in this patient group [11, 12, 17, 21, 27]. Others stated that there is a trend for longer OS and DFS in such patients [24], while others revealed a non-significant difference between both *CEBPA*-mut and *CEBPA*-wt

groups. It is of note that the absolute absence of relapse and refractoriness and the 100 % CR, OS, and DFS in our *CEBPA*-mut group may be due to low number of patients (7 patients). In addition, it was unknown if the *CEBPA*-mut patients in this study were comparable to other studies in the term of the presence of other molecular marker that may affect prognosis and disease outcome.

In summary, the incidence of *CEBPA* gene mutation in Egyptian CN-AML patients is comparable to the previously published data and it has favorable prognostic implications on the disease outcome. Fragment analysis assay is less laborious, less time consuming and more economic than sequencing and study may be suitable in screening CN-AML patients for risk stratification that will markedly affect therapeutic decisions in Egyptian AML patients. Further studies may be needed to compare both methods in parallel on large cohort of patients.

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