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Investigation of *CEBPA and CEBPA-AS* Genes Expression in Acute Myeloid Leukemia

Milad Gholami[1], Sahar Bayat[2], Saba Manoochehrabadi[1,3], Hossein Pashaiefar[4], Mir Davood Omrani[1], Hasan Jalaeikhoo[5], Vahid Reza Yassaee[6], Mohammad Reza Ebrahimpour[7], Farkhondeh Behjati*[7], Reza Mirfakhraie*[1, 6]

Abstract

Background: The pathogenicity of acute myeloid leukemia (AML) is highly influenced by genetic alterations, such as chromosomal abnormalities. Additionally, aberrations in the mechanisms involved in gene expression have been identified to have a role in the development of AML. Contradictory evidence has been reported concerning the expression of the *CEBPA* gene in AML patients. Additionally, investigation into the expression of the *CEBPA-AS* gene has yet to be explored in AML patients. The aim of the present study was to investigate the relationship between the expression of the *CEBPA* and *CEBPA-AS* genes and AML in Iranian patients.
Methods: Using quantitative real-time PCR, the expression of the *CEBPA* and *CEBPA-AS* genes was examined in the peripheral blood samples of 58 patients with de novo adult AML, and in 20 healthy controls.
Results: Overall, *CEBPA* expression analysis showed a significant up-regulation in AML patients compared with healthy controls. Interestingly, a significant up-regulation of *CEBPA* was detected in the male AML patients. Significant *CEBPA* over-expression was observed in M0 (*p-value*=0.0001), M3 (*p-value*= 0.012) and M4 (*p-value*= 0.000) FAB subtypes. Our data has also demonstrated that *CEBPA* expression is up-regulated in favorable (*p-value*= 0.006) and adverse (*p-value*= 0.042) cytogenetic risk groups. In addition, the expression of *CEBPA* was significantly increased in AML patients with an abnormal karyotype. Ectopic expression of *CEBPA-AS* was detected in seven of the AML patients.
Conclusions: Our study provides evidence for the up-regulation of *CEBPA* and the ectopic expression of *CEBPA-AS* in AML patients, suggesting that these two genes may play an important role in the pathogenesis of AML. The role of *CEBPA and CEBPA-AS* in AML patients should be further explored. This will offer potential opportunities for the development of novel treatment strategies.

Keywords: Acute Myeloid Leukemia, *CEBPA*, *CEBPA-AS*, *de novo*, Expression.

Introduction

Acute myeloid leukemia (AML) is a heterogeneous disease with considerable diversity in the molecular pathogenesis and clinical outcomes for the patient. During the progression of AML, the cancer undergoes genetic clonal complexity and clonal evolution (1-3). Acute myeloid leukemia is the most common acute leukemia in adults, accounting for approximately 80 percent of cases (4). The incidence

1: Department of Medical Genetics, School of Medicine, Shahid Beheshti University of Medical Sciences, Tehran, Iran.
2: Department of Medical Genetics, Faculty of Medicine, Tabriz University of Medical Sciences, Tabriz, Iran.
3: Hematology-Oncology and Stem Cell Transplantation Research Center, Tehran University of Medical Sciences, Tehran, Iran.
4: Department of Medical Genetics, School of Medicine, Tehran University of Medical Sciences, Tehran, Iran.
5: AJA Cancer Research Center (ACRC), AJA University of Medical Sciences, Tehran, Iran.
6: Genomic Research Center, Shahid Beheshti University of Medical Sciences, Tehran, Iran.
7: Genetics Research Center, University of Social Welfare and Rehabilitation Sciences, Tehran, Iran.
***Corresponding authors: Reza Mirfakhraie; Tel: +98 21 23872572; Fax: +98 21 23872572; E-mail: reza_mirfakhraie@yahoo.com & Farkhondeh Behjati; Tel: +98 21 22180138; Fax: +98 21 22180138; E-mail: fbehjati@gmail.com.**
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of AML increases with age, beginning from around 1.8 cases per 100,000 individuals in patients less than 65 years old. For those over 65 years, the incidence increases to 17.9 cases per 100,000 individuals (2). Although advances in the treatment of AML have led to significant improvements in the outcomes for younger patients, the prognosis in the elderly, the population that accounts for the majority of new cases, remains poor (5). Genetic alterations have been observed to hold crucial roles in the pathogenesis and the prognosis of AML (6, 7). Chromosomal abnormalities and recurrent mutations in several different genes have been detected in AML samples. The best characterized mutations are those in the fms-like tyrosine kinase 3 (*FLT3*) gene. In 20-27% of AML patients, mutations in the juxtamembrane region of the *FLT3* gene result in inappropriate gene activation (8-10).

The CCAAT enhancer binding protein alpha (*CEBPA*) is a member of the basic region leucine zipper family of transcription factors. This protein is essential for cell cycle arrest, and the inhibition of self-renewal and myeloid differentiation throughout the process of hematopoiesis (11). The expression of *CEBPA* begins when multipotent precursors commit to becoming cells of the myeloid lineage. Specifically, *CEBPA* gene expression is up-regulated during granulocytic differentiation and becomes rapidly down-regulated during the alternative monocytic pathway (12). Studies have reported genomic mutations to occur in the *CEBPA* gene among 5% to 14% of AML cases. These mutations obstruct the ability of cells to properly differentiate (11). Furthermore, the prognosis of patients with AML has been associated with *CEBPA* mRNA expression levels in patients with an intermediate-risk karyotype. Significant variabilities in the expression level of *CEBPA* has been reported among distinct cytogenetic risk groups of AML patients (13). Long noncoding RNAs (lncRNAs) have recently received widespread attention, as changes in lncRNA expression levels have been associated with various cancers (14, 15). Therefore, lncRNAs may provide a potential diagnostic or prognostic marker for specific cancers. In a recent microarray study, it was discovered that the expression levels of the lncRNA, *CEBPA-AS*, were increased in gastric cancer tissues (16).

Despite advances in our knowledge about the molecular mechanisms of AML, most patients still fall into the intermediate risk category, without a known cytogenetic or molecular aberration. The results of previous studies suggest that our understanding of AML genomics remains incomplete. Abnormalities in the expression of several different genes have been identified to contribute to the pathogenesis of AML. The expression of *CEBPA-AS* mRNA is yet to be investigated in AML patients. This is the first examination into the connection between AML and *CEBPA-AS* mRNA. Our study has examined the alternations in the expression of the *CEBPA* and *CEBPA-AS* genes in patients with AML and the relationship of this expression to the clinical and pathological characteristics of AML patients.

Materials and methods

Patients

From January 2016 to August 2017, 58 adult patients with de novo AML were collected from the Arad hospital and Namazi hospital in Iran. For the controls, 20 age and sex matched healthy donors were enrolled in the study. Written informed consent was obtained from all individuals prior to participating. Patients with antecedent hematological diseases, childhood AML and therapy-related AML were not included in our study. Patients with AML were diagnosed according to the French–American–British Cooperative Group Criteria and the 2016 revision by the World Health Organization.

The cytogenetic G-banding analysis was performed via standard methods (17). The chromosomes were interpreted according to the International System for Human Cytogenetic Nomenclature (18). This study was approved by the ethical committee of Shahid Beheshti University of Medical Sciences (IR.SBMU.MSP.REC.1396.264).

Methods

Total RNA was extracted using Trizol reagent (INTRON, Cat. 17061). DNase (GeneAll) treatment was performed to remove any contaminating genomic DNA from the RNA samples. The first-strand cDNA was synthesized from 2 µg of total RNA by using High-Capacity cDNA Reverse Transcription Kit, according to the manufacturer

instructions (ABI, Cat. 4368814). Real-time PCR analysis was conducted in duplicates using the RealQ Plus Master Mix Green (Amplicon, Cat. A323402). Reactions were run on the Rotor-Gene 6000 (Corbett Life Science) using the following primers: 5'-GCAAACTCACCGCTCCAATG-3' (forward) and 5'-TTCTCTCATGGGGGTCTGCT-3' (reverse) for CEBPA; 5'- TTCACCGACAGTGGCCTTAG -3' (forward) and 5'-CTTTACTGCGATCGTCGTGG -3' (reverse) for *CEBPA-AS*; and 5'-TGTCTTTCAGCAAGGACTGGT -3' (forward) and 5'- TGCTTACATGTCTCGATCCCAC -3' (reverse) for the housekeeping gene, beta-2-microglobulin (B2M). Each reaction was carried out in a total volume of 20 µL containing 10 pM primers, 10 µl Real-Q Plus Master Mix Green and 1µl cDNA. Thermal cycling conditions were an initial activation step for 10' at 95 °C followed by 40 cycles at 95 °C for 10 ″, 60 °C for 15 ″, and 72 °C for 20″. The thermal cycling program included 40 cycles of denaturation for 10″ at 95 °C, annealing for 15″ at 60 °C and elongation for 20″ at 72 °C.

Statistical analysis

Efficiency values for each real-time PCR were determined using the LinReg method, and the expression level of the *CEBPA* gene was estimated using REST 2009 software.

Results

The expression of *CEBPA and CEBPA-AS* transcripts was quantified from 58 AML patients (27 females and 31 males) and 20 healthy donors, ranging from the age of 15 to 82 (Table 1). According to the French–American–British classification, the AML subtypes were M0 (1.72%), M1 (18.96%), M2 (15.52%), M3 (17.24%), M4 (22.42%), M5 (10.35%), and M6 (3.44%). The FAB type was not determined in 6 (10.35%) of the patients. According to the 2017 European leukemia net (ELN) recommendations (19), the AML patients were categorized into different cytogenetic risk groups as follows: favorable 12 (20.69%), intermediate 29 (50%), unfavorable 16 (27.59%) and not determined 1 (1.72%).

Table 1. Summary of the demographic and clinical characteristics of the patients, divided by *CEBPA* and *CEBPA*-AS expression.

Variable	Total (n=58)	*CEBPA*		*CEBPA-AS*	
		Expression ratio	*P-value*	Expressed(n=7)	Not expressed(n=51)
Median age (range), years	40.88 ± 18.52 (15-82)			46 ± 13.42 (25-63)	40.88 ± 18.52 (15-82)
Age 15-60	49			6	43
Age > 60	9			1	8
Gender					
Male	31	7.18	0.006	3	28
Female	27	0.78	0.68	4	23
Female/Male		0.54	0.12		
Median of BM Blast (%)	71.27			63.43	71.53
Median WBC/µL	60986			63657	72830
FAB type					
M0	1			0	1
M1	11	14.65	0.000	2	9
M2	9	0.95	0.94	1	8
M3	10	0.000	0.97	0	10
M4	13	3.26	0.012	1	12
M5	6	8.37	0.000	1	5
M6	2	1.03	0.99	0	2
M7	0	1.5	0.12	0	0
Not determined	6			2	4
Cytogenetics risk group					
Favorable	12	4.43	0.006	2	10
Intermediate	29	1.97	0.17	4	25
Unfavorable	16	3.16	0.042	1	15
Not determined	1			0	1
Karyotype					
Normal	21	1.31	0.614	4	17
Abnormal	36	5.53	0.001	3	33

Overall, the expression level of *CEBPA* was significantly up-regulated inpatients with AML compared to the healthy donors (*p-value* = 0.027). When examining the different sexes, the expression of *CEBPA* was observed to be significantly higher in the male AML patients (*p-value*=0.006). According to the FAB type classification, significant over-expression was observed in the M0, M3 and M4 subtypes (*p-value*=0.0001, *p-value*=0.012 and *p-value*=0.000 respectively). According to the cytogenetic risk groups, *CEBPA* was statistically over-expressed in the favorable and unfavorable groups (*p-value* = 0.006 and *p-value* = 0.042, respectively). In addition, the expression level of *CEBPA* was significantly increased in AML patients with an abnormal karyotype (*p-value*=0.001). Furthermore, the expression of *CEBPA* was not significantly different based on the mutation status of the FLT3 gene.

Interestingly, in the healthy controls, *CEBPA-AS* was not observed to be expressed. However, in 7 of the AML patients (both males and females), ectopic expression of *CEBPA-AS* was observed (Table 1). The expression of *CEBPA-AS* was detected in M1, M2, M4 and M5 groups and in all cytogenetic risk groups.

Discussion

The identification and characterization of genomic alterations in AML patients, such as chromosomal abnormalities and recurrent mutations in the FLT3, NPM1, DNMT3A and IDH1 genes have provided important insight into the pathogenesis of AML (20). However, recent studies have shown that many patients with AML carry no mutations in any of the currently recognized driver genes associated with AML pathogenesis (21). At the present time, none of the current classification systems are entirely accurate. Therefore, expression analysis is necessary to enable better classification, risk assessment, and therapeutic approaches for patients with AML.

In the present study, our results show CEBPA to be significantly up-regulated in AML patients. Additionally, we observed associations between CEBPA gene expression and sex and, FAB type (M0, M3 and M4) and cytogenetic risk group (favorable and unfavorable). Our results suggest that CEBPA may play an important role in the development and progression of AML. Conflicting results have been obtained from previous studies concerning the expression of CEBPA in AML patients (22-25).

Pabst et al has shown that the expression of the *CEBPA* gene was significantly down-regulated in AML patients classified as the FAB-M2 subtype and t(8;21)(q22;q22) karyotype (22). However, a separate study reported there to be increases in the expression of *CEBPA* in a large number of patients with AML. In those patients with decreased levels of *CEBPA* expression, the prognosis was relatively poor (23). Another study by D'Alò et al reported no significant alterations to the gene expression of *CEBPA* in AML patients. However, a significant increase was observed in patients positive for the myeloid differentiation markers, CD33 and CD11c. Furthermore, in AML patients with leukopenia, a lower level of *CEBPA* expression was observed (24). Grossmann et al has previously reported *CEBPA* to be down-regulated in RUNX1-mutated AML harboring t(8;21), whereas t(15;17)/PML-RARA-mutated cases showed enhanced *CEBPA* expression (25).

The integration of a whole genome approach including non–coding RNAs may lead to an improved understanding of AML pathogenesis. Alterations in the expression level of lncRNAs have been increasingly reported in different types of cancer, suggesting an important role in carcinogenesis. These molecules may therefore be considered as novel targets for cancer diagnosis and treatment (16). This is the first study to investigate the expression of *CEBPA-AS* in AML cases. We have shown that the *CEBPA-AS* gene was not expressed in healthy controls, although ectopic expression of *CEBPA-AS* was observed in 7 of the AML patients. The expression of *CEBPA-AS* was previously investigated in gastric cancer, and it was shown that the expression of this lncRNA was increased in tumor tissues (16).

In conclusion, the expression of *CEBPA* and *CEBPA-AS* may hold the potential as a marker for AML prognosis. Our results highlight the importance of *CEBPA* and *CEBPA-AS* in AML pathogenesis. Further studies are required in order to confirm these findings and the importance of these genes in the classification and treatment of AML.

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The authors have no conflict of interest to declare.

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