Original article:

Study on the significance correlation between CEBPA and Calreticulin at mRNA level diagnosed in de nevo AML patients

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Abstract:

Objectives: Calreticulin (CALR) and CCAAT/enhancer binding protein (C/EBP) alpha (CEBPA) are two important multifunctional proteins which play different roles in regulation of hematopoiesis. Expressional changes of these genes were related to the malignancy. **Methods:** The present study aimed to investigate the expression levels of CALR and CEBPA genes in 96 de novo AML patients compared to 18 normal people as the control group through the Real-Time-PCR. **Results:** Results of the present study revealed that the expression of these genes was significantly higher in patients with AML than the normal group (P <0.0001). Furthermore, there was a significant and positive correlation between CALR and CEBPA (P= 0.001 and r= 0.348). **Discussion:** Higher level of CALR expression was expected, but the over-expression of CEBPA was on the contrary to its well-known role in the myeloid maturation. Based on the studies, CALR probably played roles in the expression of oncogenic CEBPA and it repressed the CEBPA translation in tumor suppressor gene (TSG). **Conclusion:** The present study first indicated the over-expression of CALR in AML patients and compared it with the healthy normal control group and also found a positive relationship between CALR and CEBPA expression in the AML patients.

Keywords: Acute myeloid leukemia (AML), CEBPA, CALR, Oncogene, Malignancy

1. Introduction

Calreticulin (CALR) is a multifunctional protein which is involved in regulation of intracellular calcium homoeostasis, protein synthesis, and cell adhesion and plays role as a chaperone[1, 2] .CALR contributes to the development of cancer through multiple paths and ultimately it can lead to the increased expression, migration and survival of cancer cells. Over-expression of this gene can be seen in multitude of solid tumors and it is associated with more advanced and aggressive diseases[1-4]. The mutation of this gene was also seen in 15-25 percent of ET and MPL patients, but there was not any other common mutation such as JAK2[5]. A few studies have

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investigated the relationship between expression of this gene in the AML patients and changed expression of this gene in the AML patients compared to other hematological malignancies[6-12].

In addition to the roles of CALR in calcium storage, it can be as a RNA binding protein. Therefore, since the CCAAT enhancer binding protein alpha (CEBPA) mRNA has the GC-rich regions, CALR interacts with this section[11,13]. CEBPA is a single Exon gene located on the chromosome 19 q13.1 with the length of 2783 bp. This gene belongs to the basic region leucine zipper (bZIP) family[14,15]. Its mRNA is translated to two protein isoforms using the alternative AUG initiation codons: a full-length isoform of 42 kDa and an N-terminal truncated form of 30 kDa[16]. P30 proteins fail to induce granulocyte differentiation and block anti-proliferative activities of the full-length p42 isoform [17,18]. According to the in-vitro studies, translation of CEBPA mRNA can be specifically blocked by CALR binding. In this regard, CEBPA can be translated by mutation or deletion in the rich-GC site of CALR. Other studies found that when CALR is over-expressed the inhibiting CEBPA causes progression in the cell cycle leading to leukemia by blocking the CEBPA ability in regulation of downstream genes[13].

The CALR expression model has not been evaluated in AML patients compared to healthy group and also different subtypes of AML yet. Since the CALR and CEBPA are involved in the leukemogenesis, and the relationship between these genes is obvious at the protein level, the present study evaluated the correlation of these genes at the mRNA level in the AML patients and its subgroups.

2. Method

2.1. Patient samples

We provided 98 samples of bone marrow and peripheral blood from the newly diagnosed AML patients and 18 samples from the healthy control group who were referred to Mofid and Imam Khomeini Hospitals of Tehran, Iran, from 2012 to 2014. Patients' mean age was 47 years (ranged from 2 to 87 years) and they were 44 females and 52 males. Numbers of patients in each various morphological subtype of FAB/WHO were as follows: 10 patients with M0; 20 patients with M1; 14 patients with M2; 30 patients with M3; 14 patients with M4; 7 patients with M5 and a patient with M6.

2.2. RNA isolation, cDNA synthesis and Real-Time polymerase chain reaction (qRT-PCR)

We extracted the total cellular RNA from the bone marrow and peripheral blood through the kit Rneasy (Qiagen, Germany); and the RNA quality was detected by a Nanodrop (Thermo scientific, USA), All samples had a high purity (OD 260/280 nm ratio >1.8). 0.5 mg of RNA was subsequently transcribed into cDNA with a final volume of 20 μL through a thermo scientific kit (USA). cDNA with a 20μL of final volume was synthesized by means of a cDNA synthesis kit (Thermo Scientific, USA) and finally an aliquot of 1/10th of cDNA (1μL) was applied as a substrate for qRT- PCR amplification[19].

We designed primers through Oligo 7.56 software and NCBI-Blast site (Table 1), and analyzed the CALR, CEBPA and ABL mRNA expression in patients and the healthy control group by qRT-PCR (Rotor-Gene 6000, Bosch). A standard curve was produced for each of qRT-PCR reactions through five consecutive 1:10 cDNA dilution samples (1, 0.1, 0.01 and 0.001). Components of the qRT-PCR reaction for each target consisted of forward and reverse primer, 1 μL of template cDNA, 6 μL water, and μL of RealQ Plus 2x Master Mix Green-Low ROX (Ampliqon, Denmark) for a total reaction volume of 15 μL.

Table 1. Nucleotide sequences of primers used for ABL,CEBPA and CALR qRT-PCR reactions

Gene	Forward	Reverse
ABL	AGTCTCAGGATGCAGGTGCT	TAGGCTGGGGCTTTTTGTAA
CALR	GGTGCAGTTCACGGTGAAAC	GTCTCCGTGCATGTCTGTC
CEBPA	TTGTGCCTTGGAAATGCAAAC	TCGGGAAGGAGGCAGGAAAC

For each reaction, the thermal cycling conditions included an initial maintenance at 95°C for 10 minutes followed by 40 cycles of primary denaturation at 95°C for 10 seconds and also the annealing/extension at 65°C for 15 seconds and a final extension at 72°C for 10 minutes. Duplicate Ct was obtained for all patients; and the relative amount of mRNA expression was calculated for all samples (fold change = FQ) through the LIVAK method (2-ΔΔct) [15, 20-22].

2.3. Statistical analysis

Statistical analysis was done by SPSS 16.0 and GraphPad Prism 6.07 software. Results were presented as the mean± standard error of the structural equation modeling (SEM) (SEM) and P-value of 0.05 and under. LOG was used for normalization of 2-Δct statistical data in SPSS. Shapiro-Wilk and Kolmogorov-Smirnov tests were used to evaluate normal distribution of data. Furthermore, t-test was used to determine significant differences between AML patients and healthy control group in terms of CALR and CEBPA expression. Pearson's chi-squared test was used to measure the linear correlation between CEBPA and CALR.

3. Results

3.1. CALR and CEBPA expression is significantly higher in healthy control group than the AML patients

We measured the mean CT value for the housekeeping gene of ABL in AML patients and healthy control group at 27.45± 0.33 and 29.10± 0.31, respectively, and also for the target gene of CALR at 22.16 ± 0.29 and 27.20 ± 0.23, respectively. We defined the CALR expression level of 1.30-5 at the confidence interval of 95% as a normal or intermediate expression level for a healthy population. According to this range, patients were divided into three groups; and the patients at the CALR expression level were put at the normal range (11 out of 96 AML patients) and had an intermediate expression level while those with levels above 5.46 to 240.471 (72 out of 96 patients) were considered as the patients with high expression; and those with levels under 1.30 to 0.0269 (13 out of 96 patients) were considered as the patients with low expression. In the present study, a majority of patients had high expression levels. 9.14 FOLD increased according to the results of CALR gene expression in patients compared to healthy control group. T-test statistical analysis indicated that there was a significant difference between two groups in terms of expression levels for both CALR and CEBPA (P<0.0001) and (P<0.0001) respectively (Figure 1-A). The average expression levels (±SD), which were measured for AML patients and normal control were 9.23±2.76 and 1.69±0.42 respectively for CEBPA (Figure 1-B).
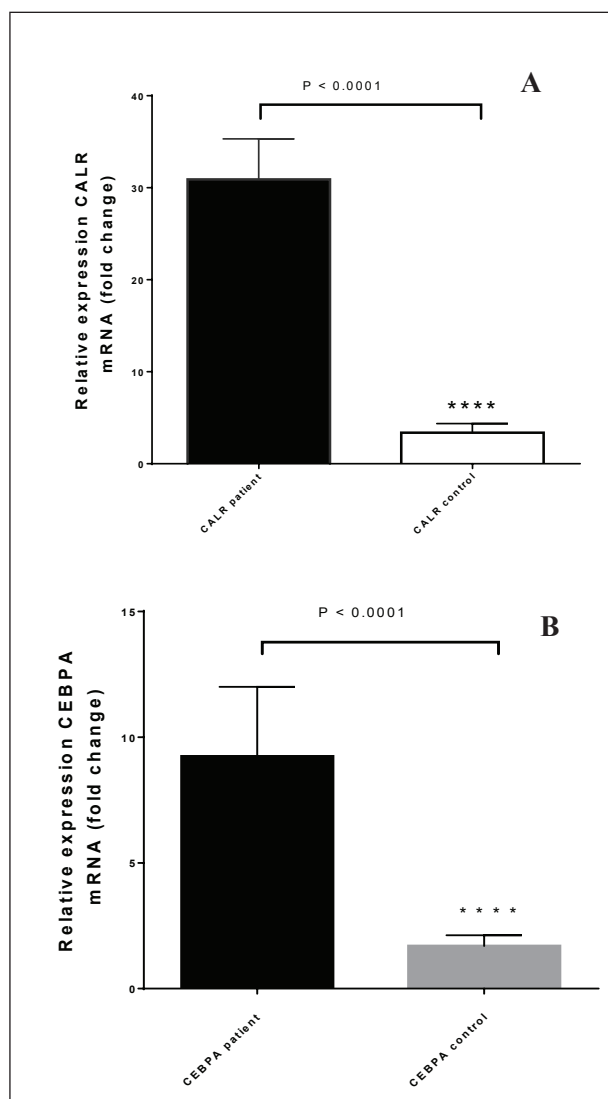

Figure 1. Relative expression of CEBPA and CALR in 96 AML patients and 18 healthy patients was measured from Ct values and normalized against a reference gene (ABL). A) A significant difference (P<0.0001) between CALR expression in AML patients and healthy patients was identified. A relative CALR expression level of 3.09 ± 4.41 (SD) was measured in AML patients in comparison to 3.38 ± 0.98 (SD) in the normal control group .B) A significant difference (P<0.0001) between CEBPA expression in AML patients and healthy patients was also identified. A relative CEBPA expression level of 9.23 ± 2.76 (SD) was measured in AML patients in comparison to 1.69 ± 0.42 (SD) in the normal control group..

This comparison was separately evaluated in patients and control group in samples of peripheral blood (8 peripheral blood samples of control group and 28 peripheral blood samples of patients) and samples of bone marrow, 10 samples of BM from the control group

and 68 samples from the BM patients). The obtained results indicated the consistency with all results (combination of two samples) (Figure 2, A & B).
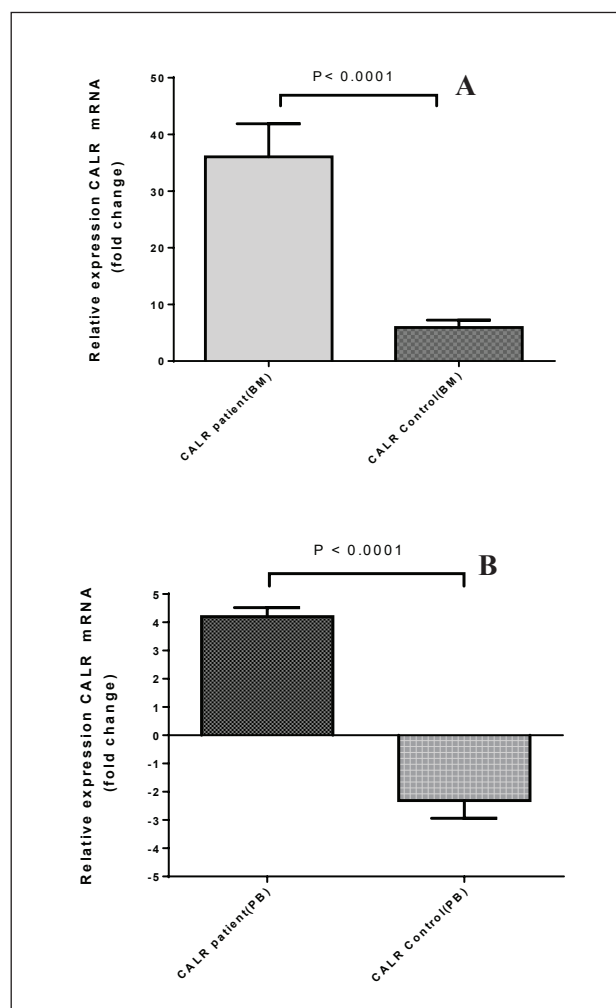

3.2. Differential expression of CALR in AML FAB subtypes

Differential expression of CALR between different FAB subtypes, which were expressed in AML patient samples, was assessed by the ANOVA. There was not any statistical significant difference between different FAB subgroups. The lowest and highest expression of CALR (±SEM) was seen in M3 (47.58 ± 1.09) and M0/M1/M2 (3.38 ± 0.98) respectively. However, The M0/M1/M2, M4/M5 and M3 subgroups had significant over-expression compared with the normal control group (P<0.0001, P<0.0001 and P=0.002 respectively), but there was not any correlation between these subgroups (Figure 3).

Figure 2. The relative expression of CALR were evaluated between AML patients and healthy controls group in samples of peripheral blood (PB) (8 healthy control group and 28 AML patients) and bone marrow (BM) (10 healthy control group and 68 AML patients). A) A significant difference (P<0.0001) between CALR expression in AML patients and healthy control group with BM samples was identified. A relative CALR expression level of 36.05 ± 5.93 (SD) was measured in AML patients with BM samples in comparison to 5.93 ± 1.29 (SD) in the normal control group with BM samples. B) A significant difference (P<0.0001) between CALR expression in AML patients and healthy control group with PB samples was also identified. A relative CALR expression level of 18.39 ± 4.57 (SD) was measured in AML patients with PB samples in comparison to 0.20 ± 0.07 (SD) in the healthy control group with PB samples.
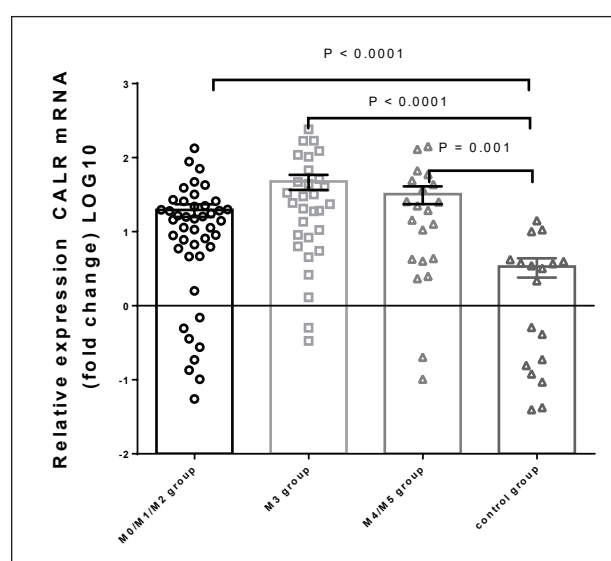
Figure 3. The relative expression of CALR measured in 96 AML patients are grouped by their corresponding FAB subgroup and analysis by means of the ANOVA test determines that there is no significant difference in expression between these subgroups The highest relative expression of CALR was measured at 47.58 ± 1.09 (SEM) in the M3 subgroup and the lowest at 3.38 ± 0.98 (SEM) in the M0/M1/M2 subgroup. TheM0/M1/M2, M4/M5 and M3 subgroups have significant overexpression in comparison to the healthy control group (P P<0.0001, P<0.0001 and P=0.002, respectively) but there is no significant difference in CALR expression between these subgroups.

3.3. Correlation between the CEBPA and CALR expression levels

We carried out an analysis by means of the statistical test in order to identify any correlation between expression of CEBPA and CALR genes in AML patients. According to the analysis, there was a

positive and significant correlation between CEBPA and CALR (P= 0.001 and r= 0.348) in AML patients, and thus there was a relationship between their expression. (Figure 4)
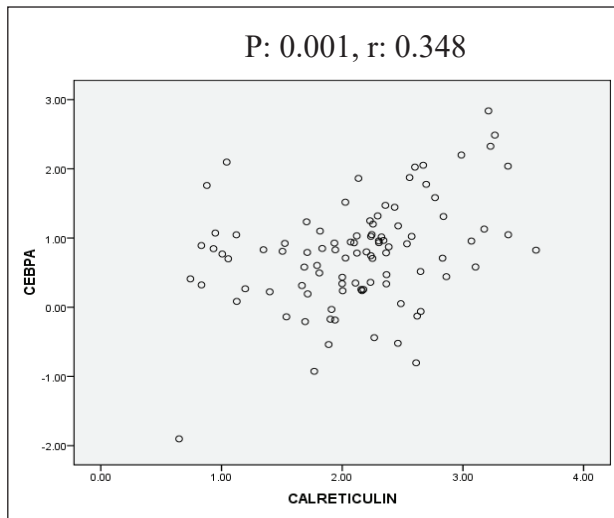

Figure 4. Statistical analysis by means of Pearson's chi-squared test reveals dependence and relation between the expression of CEBPA and CALR. Correlation between CEBPA and CALR in 96 AML patients was determined to be positive and significant (P= 0.001, r= 0.348).

4. Discussion

According to our study, the CALR was expressed at higher levels in patients with AML compared with the healthy normal control group (9.14 fold change for CALR). Different FAB subtypes indicated over-expression compared to the healthy normal control group. A positive correlation was also observed between CALR and CEBPA mRNA levels. This correlation was stronger in patients with minimal differentiation (non-m3) than the maximal differentiation (m3).

In a research on patients with progressive breast cancer the CALR over-expression was consistent with our findings[23]. Expression of CALR in solid tumors was associated with the malignancy. According to reports of various studies, the expression level of this gene is higher in cancerous tissues than normal tissues. We assumed that the CALR played roles in development of most cancers through several different functional paths and finally led to the increased proliferation, migration and survival of cancer cells[16,24,25]. In general, this gene plays role in onset and progression of cancer and can be considered as a prognostic marker[26]. Other studies on the CALR mRNA levels in the AML patients indicated higher levels of CALR gene than other hematologic malignancies such as the ALL and MPN[12].

Most of the previous studies on CALR in solid tumors and leukemia are consistent with our results. Moreover, we found an increase in the expression of CALR patients with AML due to its role in inhibiting the CEBPA (it increased proliferation and reduced differentiation). The increased expression of this marker probably leads to the oncogenic behavior. In the present study, the obtained data indicated a significant correlation between the CALR and CEBPA expression in all AML cases (P=0.001, R= 0.348). According to a research by Daniel Helbling et al (2005), the CBFB-SMMHC fusion protein is correlated with the increased CALR expression; and CALR leads to the suppressed CEBPA expression by inhibiting CEBPA. The CALR expression block resulted to CEBPA expression retrieval in patients with this fusion. The present study also indicated the over-expressed CALR in AML patients with inv(16) and the activation of the CALR-induced repression of CEBPA in AML patients[10,11].

Based on these studies, the CALR can be as an operating loss of CEBPA functional protein and raise Protein of 30 kDa in spite of 42 kDa, and thus, a similar mechanism with increased CALR expression can increase the CEBPA expression until it is converted to 30 kDa protein which forms the oncogenic transcription factor according to the present study[27] Timchenko LT et al (2002) found that when CALR is over-expressed, it can result in the inhibited CEBPA, and thus it can cause progression in the cell cycle and block the CEBPA ability in the regulated downstream genes and thus it can cause the leukemogenesis[13]. Therefore, our results suggested that there was an over-expression of CALR in the malignancy leading to the inhibited translation of 42-kDa in CEBPA, while 30-kDa of CEBPA was strengthened.

5. Conclusion

In the present study, the expression of CALR and CEBPA genes was significantly higher in AML patients than the healthy control group. We expected this over-expression of CALR, but the over-expression of CEBPA was unexpected (CEBPA is probably converted to oncogenic form of "possibly over-expressed 30 kDa isoforms" in the malignancy[28]. In addition, the over-expression of these genes at the mRNA level was in the same direction probably due to the role of CALR in the expression of oncogenic form of CEBPA and repression of translation of isoforms of the tumor suppressor gene (TSG). The present

study initially indicated the over-expressed CALR in the AML patients compared to the healthy group and also found a functional relationship between CALR and CEBPA in the malignancy. There a positive correlation between their mRNA le which could explain a simultaneous increase in expression of these two genes in the AML pati compared to the healthy control group. Future stu are suggested evaluating the expression of 30 42 kDa CEBPA isoform protein simultaneous ..... CALR as well as evaluating the positive correlation with the exact mechanism between these two gene at the mRNA levels. It is also suggested evaluating the prognosis in patients with these genes when they are simultaneously over-expressed.

Abbreviations:
CEBPA, The CCAAT/Enhancer Binding Protein (C/EBP), Alpha ; CALR, calreticulin; AML, Acute Myeloid Leukemia; HSCs, Hematopoietic stem cells; BM, Bone Marrow; PB, peripheral blood; S.E.M,standard error of the mean.

Declarations:
Consent for publication:
All participants in the study signed a written consent form to permit publication of the individual data.

Competing interests:
The authors declare that they have no competing interests.

Authors' contributions:
All authors contribute in this work and all authors read and approved the final manuscript.

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