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# Analysis of EVI1 Gene Expression in Acute Myeloid Leukemia Patients in the Northeast of Iran

**Mohammad-Hadi Sadeghian, Hosein Ayatollahi, Seyyede-Fatemeh Shams, Zahra Rezaei Dezaki♦**

*Department of Pathology, Cancer Molecular Pathology Research Center, Mashhad University of Medical Sciences, Mashhad, Iran*

## Abstract

**Background:** Acute myeloid leukemia (AML) is a heterogeneous disease characterized by increasing immature blood cells in the bone marrow. Aberrant expression of specific genes is a common finding in AML. The proto-oncogene EVI1 is located in a 3q26 position that encodes a zinc finger protein. In the present research, we analyzed the expression of EVI1 gene in 88 Iranian patients with AML.

**Method:** This case-control study was performed in the Cancer Molecular Pathology Research Center of Mashhad University of Medical Sciences. Total RNA was extracted from the bone marrow or peripheral blood mononuclear cells of 88 AML patients and the same number of healthy cases. The expression level of EVI1 was investigated by real-time polymerase chain reaction (RT-PCR). Relative quantification analysis was performed by comparative CT ($2^{-\Delta\Delta CT}$) method.

**Results:** AML patients expressed a high level of EVI1 (32.95%). In addition, a statistically significant relationship was detected between t (8;21) and EVI1 expression ($P$=0.049). No correlations were found between EVI1 expression and FAB (French American British) classification, and also between FLT3-ITD mutation and white blood cell count ($P$ =0.6). Furthermore, no correlations were found between the level of EVI1 expression and overall survival ($P$=0.72).

**Conclusion:** Although EVI1 is highly expressed in peripheral blood mononuclear cells of AML patient, it cannot be considered as an independent prognostic factor.

***Keywords:*** EVI1 Gene, Acute myeloid leukemia, Quantitative real-time PCR

♦Corresponding Author:
Zahra Rezaei Dezaki, MSc.
Cancer Molecular Pathology Research Center, Mashhad University of Medical Sciences, Mashhad, Iran
Tel: +985138012584
Email: Zahra.rzi69@gmail.com

## Introduction

Acute myeloid leukemia (AML) is a heterogeneous blood malignancy characterized by recurrent genetic anomalies. It is theorized that AML results from cooperative but functionally distinct (epi) genetic aberrations.[1,2] AML accounts for less

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than 1% of all malignancies and about 25% of leukemia cases. It is usually more prevalent in adults and the incidence increases with age.[3] Improper expression of specific genes is a common finding in AML and may induce clinically relevant biological subsets.[1] Ecotropic virus integration site 1 (EVI1) is a proto-oncogene that operates as a transcriptional regulator of self-renewal hematopoietic stem cell.[4-6] EVI1 is one of the protein isoforms encoded by the MDS1 and EVI1 Complex Locus (MECOM) that is located on the 3q26 position. MECOM encodes several spliced transcripts including MDS1-EVI1, MDS1, and EVI1.[7] EVI1 encodes a nuclear DNA binding protein with two zinc-finger domains; i.e., the N-terminal domain and C-terminal that contain 7 and 3 zinc fingers, respectively.[8,9] This regulator binds to a number of transcription factors and epigenetic regulators. Some studies have shown that EVI1 block TGF-beta and IFN-alpha pathways by changing the function of transcription factors such as GATA1 and RUNX, and cooperating with co-activators and co-repressors of genetic transcription.[10,11] Moreover, a recent study showed that EVI1 may regulate the cell cycle, proliferation, differentiation, and apoptosis. Also, high expression of EVII may contribute to aggressive progress of disease via transcriptional repression of membrane-spanning-4-domains subfamily-A member-3 (MS4A3) and suppression of microRNAs 133 and 431 in AML and other cancers.[12-14]

High expression of EVI1, through 3q26 anomalies such as t (3; 3) (q21; q26) or inv3 (q21; q26) has been implicated as effective markers in the progress or evolution of high-risk AML. Nevertheless, it has been reported in AML patients without chromosomal rearrangements including 3q26/EVI1 locus.[15, 16]

EVI1 overexpression has been detected in 10%

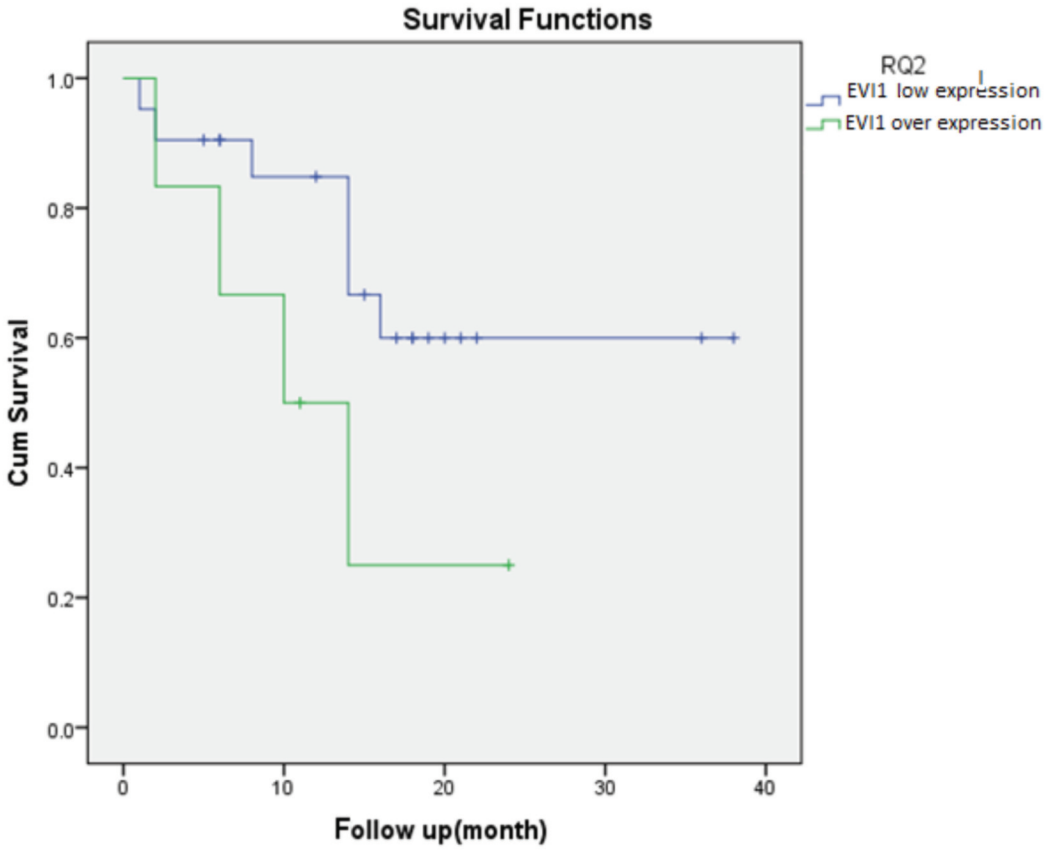


**Figure 1.** Survival analysis of a series of AML patients according to EVI1 expression status. Kaplan-Meier analysis showed a lower overall survival in patients with EVI1 overexpression comparison to patients with low EVI1 Expression.

**Table 1.** CBC Index results in two groups of overexpression and low expression of EVI1

| CBC Index | Overexpression SD±Mean | Low expression SD±Mean | *P*-value |
|---|---|---|---|
| WBC | 27.9 ± 61.33 | 25.5 ± 55.07 | 0.68 |
| RBC | 2.8 ± 0.9 | 2.9 ± 0.90.48 | |
| HGB | 6.95 ± 3.05 | 7.65 ± 2.88 | 0.11 |
| HCT | 22.9 ± 7.0 | 25.5 ± 8.1 | 0.14 |
| MCV | 87.25 ± 13.45 | 88.7 ± 8.6 | 0.43 |
| MCH | 28.2 ± 3.4 | 29.4 ± 3.2 | 0.10 |
| MCHC | 31.9 ± 2.6 | 32.6 ± 2.2 | 0.17 |
| PLT | 54.5 ± 132.5 | 44.5 ± 64.1 | 0.07 |

WBC: White Blood Cell, RBC: Red Blood Cell, HGB: Hemoglobin, HCT: Hematocrit, MCV: Mean Corpuscular Volume, MCH: Mean Corpuscular Hemoglobin, MCHC: Mean Corpuscular Hemoglobin Concentration, and PLT: platelet

of de novo AMLs without chromosome rearrangements involving 3q26.2; however, it was not related to shortened survival of AML patients.[17] Barjesteh et al., Lugthart et al., and Qin Y.Z. et al. identified no relationship between EVI1 gene expression and FAB classification.[15,18,19] In comparison, in subsequent studies by Groeschel et al., it was reported that M4 subtype of FAB classification had the highest prevalence of EVI1 expression.[20] Guangsong et al. and X-He et al. reported the highest prevalence of the gene expression in AML-M3 and AML-M5, respectively.[21,22]

Some studies did not detect any significant differences between FLT3 mutation and EVI1 expression rate[18,21,23] but some other reports demonstrated an inverse correlation between EVI1 expression level and FLT3 mutation.[15,19,20]

Although X-He et al. identified no relationships between EVI1 expression rate and NMP1

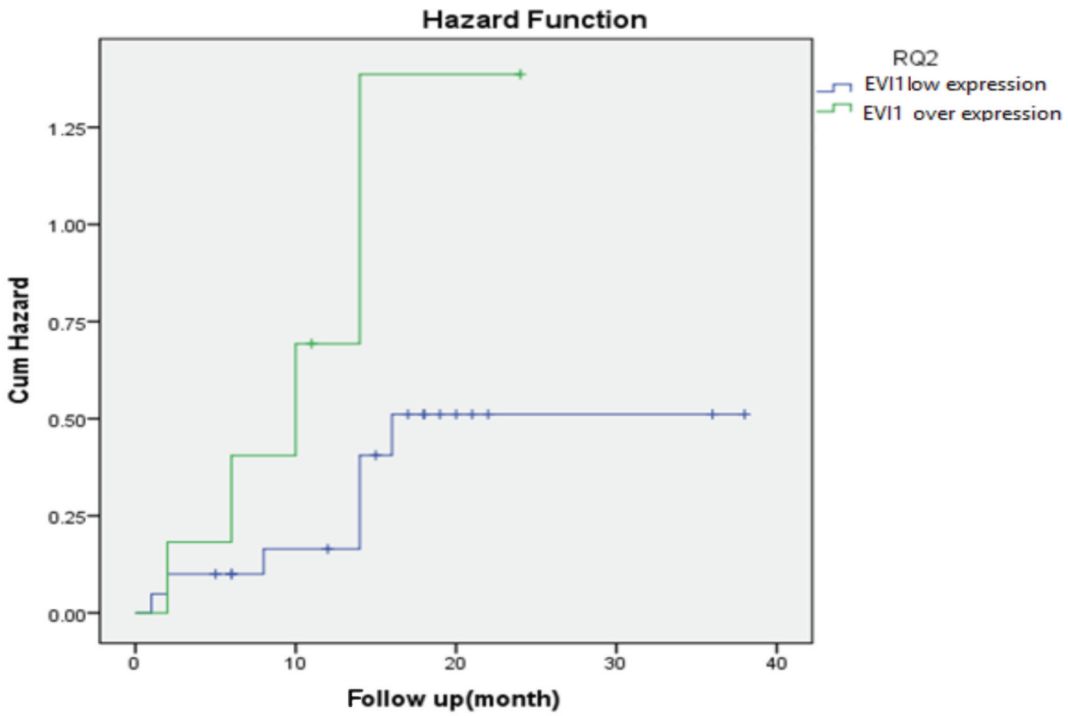


**Figure 2.** Hazard analysis of a series of AML patients according to EVI1 expression status. Kaplan-Meier analysis showed an upper Hazard ratio in a patient with EVI1 overexpression comparison to the patient with low EVI1 expression.

**Table 2.** Comparison of the clinical and laboratory data between two groups of patients: overexpressed and low expressed EVI1

| | | EVI1 over expression | EVI1 low expression | *P*-value | |
|---|---|---|---|---|---|
| **Gender** | Male | 16 (55.2%) | 29 (49.2%) | 0.59 | |
| | Female | 13 (44.8%) | 30 (50.8%) | | |
| **WHO Classification** | t(8;21) | 0 (0.0%) | 8 (13.6%) | 0.049 | 0.04 |
| | t(15;17) | 5 (17.2%) | 18 (30.5%) | 0.18 | |
| | Inv(16) | 1 (3.4%) | 4 (6.8%) | 0.999 | |
| | AML with NPM1 mutation | 1 (3.4%) | 3 (5.1%) | 0.999 | |
| | NOS | 22 (75.9%) | 26 (44.1%) | 0.005 | |
| **FAB Classification** | M0 | 5 (17.2%) | 4 (6.8%) | 0.58 | 0.31 |
| | M1 | 2 (6.9%) | 6 (10.2%) | 0.23 | |
| | M2 | 8 (27.6%) | 15 (25.4%) | 0.78 | |
| | M3 | 5 (17.2%) | 18 (30.5%) | 0.67 | |
| | M4 | 4 (13.8%) | 12 (20.3%) | 0.76 | |
| | M5 | 4 (13.8%) | 4 (6.8%) | 0.26 | |
| | M7 | 1 (3.4%) | 0 (0%) | - | |
| **FLT3 mutation** | Positive | 3 (16.7%) | 8 (24.2%) | 0.7 | |
| | Negative | 15 (83.3%) | 25 (75.8%) | | |

Abbreviations: FAB: French American British, FLT3 mutation: FMS-like tyrosine kinase 3 mutation, NPM1 mutation: Nucleophosmin 1 mutation, NOS: Not Otherwise Categorized, CEBPA mutation: Mutations in CCAAT/enhancer binding protein α, PLT: Platelet. There is no correlation between gene expression level and FAB subtype, Favorable risk chromosomal abnormality, FLT3 mutation, NPM1 mutation, CEBPA mutation, and PLT count.

mutation,[21] other studies reported an inverse correlation between EVI1 rate and NMP1 mutation.[19,20,23] Most studies stated that EVI1 gene expression reduced the survival of AML patients,[15,18,20,21,23,24] but some others found no effect on overall survival.[25,26] Therefore, the predictive effect of gene expression is still controversial and indicates the necessity of further studies.

Considering that few studies have been conducted on EVI1 gene expression level and the results are controversial, the present study was conducted to examine the rate of EVI1 gene expression in AML patients in the Northeast of Iran.

## Patients and Methods

This case-control study was done in Ghaem University Hospital of Mashhad University of Medical Sciences (MUMS). The study was approved by the ethics committee of MUMS (committee code: IR.MUMS.fm.REC.1395.404).

A total of 88 AML patients at the time of diagnosis and 88 healthy age and sex-matched volunteers were assessed. Bone marrow and peripheral blood samples of recognized AML patients and controls were collected in Ethylene Diamine Tetra Acetic acid-K2 (EDTA-K2).

Patients were categorized according to French-American British (FAB) and the World Health Organization (WHO) criteria. AML was diagnosed by two expert hemato-pathologists.

Acute leukemia was diagnosed as the existence of at least 20% blasts in peripheral blood or bone marrow smears, which were positive for myeloperoxidase or Sudan Black B staining and myeloid markers including CD33, CD13, CD117, CD64, and CD14. Other stainings such as periodic acid Schiff were similarly performed when needed. Complete blood count (CBC) of all patients was available. AML patients under treatment or already treated, as well as patients with indefinite diagnosis and improper samples were excluded from the study.

### *RNA extraction and cDNA synthesis*

RNA was extracted from blood and bone marrow PBMC using the Tri-pure solution RNA blood kit (Roche, Germany). Contaminating genomic DNA was digested with DNase-I (Thermo, USA), and cDNA was synthesized using random hexamer primers and M-MLV reverse

**Table 3.** Means of survival time in AML patients with over and low expression of EVI1

| | SD | Overall survival (OS) Log rank P | OS(95% interval) | *P*-value |
|---|---|---|---|---|
| EVI1 over expression | 3.32 | 12.5 | 5.9-19.01 | 0.081 |
| EVI1 low expression | 3.30 | 26.99 | 20.52-33.47 | |
| Overall | 3.02 | 24.52 | 18.5-30.45 | |

Patients with overexpression of EVI1 had lower OS but it is not significant.

transcriptase according to the manufacturers' protocols. The effectiveness of the DNase-I treatment was verified by PCR method. Glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene was applied as a housekeeping gene.

### *Reverse transcriptase quantitative polymerase chain reaction (RT-PCR)*

RT-PCR was performed using the SYBR Green method (ABI step one, USA). Each reaction contained 2 µL of synthesized cDNA, 200 nM of each forward and reverse primers, and 12.5 µL 2× PCR master mix. PCR steps and conditions were as follows: denaturation at 95℃ for 10 min, 40 cycles of 95℃ for 15 s and 60℃ for 1 min. Primers were designed according to the study of Guangsong et al.[22] The forward and the sequences of the reverse primers of the EVI1 gene were 5′-GAGAGCAGCCTTACAGAT-3′ and 5′-GACATGTTCCCATTCTCATGA-3′, respectively. In addition, the sequences for forward and reverse primers of GAPDH were 5′- CCAA GGTCATCCATGACAAC-3′ and 5 ′-CCATCACGC CACAGTTTC-3, respectively. The comparative delta-delta Ct method was used to detective relative expression levels of the EVI1.[27]

Based on the relative expression level, values higher than 1 were considered as overexpression and those lower than 1 or undetectable were considered as EVI1 low expression.

## Statistical analysis

Statistical analysis was performed using SPSS version 22 (version 22, SPSS Inc) software. The Kolmogorov-Smirnov test was used to evaluate the normality of the data distribution. Independent sample t-test and Mann-Whitney were used for comparing continuous variables between the groups. Independent sample t-test was applied to compare the mean of EVI1expression between patients and control group. Pearson analysis and Fisher exact test were performed to evaluate the correlation between the level of EVI1 expression and molecular, demographic (sex and age), and laboratory data of studied subjects. The Kaplan-Meier/Log rank method was used to estimate overall survival (OS). OS was described as the time of patient's entry to study to death time due to any causes. For all analyses, statistical significance was defined as a *P*-value less than 0.05.

## Results

### *Correlation between EVI1 expression and Patients Characteristics*

De novo AML and healthy control samples were subjected to RT-PCR to determine the expression level of EVII gene.

In this study, 45 (51.1%) of patients were male and 43 (48.9%) were female. In terms of sex distribution, 47 (53.4%) and 41 (46.6%) of the participants were male and female, respectively, in the control group. The average age±SD of patients and controls was 28.58±20.21 and 28.47±19.76 years, respectively.

There were no statistically significant differences between the AML patients and the control group in sex and age (*P*=0.76 and *P*=0.97), respectively.

The mean relative expression of EVI1 was 3.46±4.2 (range: 0.6-7.6) in patients. Patients were categorized into two groups based on EVI1 expression level: a group with higher expression and the other with lower expression of EVI1.

It was observed that 29 out of 88 patients' samples (32.95%) had EVII overexpression, and 59 out of 88 samples (67%) had EVII low expression; however, there was no correlation

between the level of EVI1 gene expression and the age (*P*= 0.48) or gender (*P*=0.59) of patients.

The correlation between CBC indices and the EVI1 expression level was investigated. There were a higher platelets count in cases with overexpression of EVI1; but no more relationships were found in other CBC indices with EVI1 expression (Table 1). Significant differences were found between two groups concerning WHO classification. In comparison, no differences were observed between FAB-AML subgroups and the level of EVI1 expression (Table 2).

### *EVI1 Overexpression and Molecular Parameters*

Cytogenetic and molecular data of patients were compared between EVI1 overexpressed cases and low-expressed ones. No significant difference was found between two groups from the point of FLT3-ITD mutation (*P*= 0.72). As shown in table 2, the frequency of molecular markers based on WHO classification was significantly different between the two groups (*P*= 0.041). Among the AML patients, the most common subtypes of the WHO classification were NOS type with a frequency of 54.54%. Patients with EVI1 overexpression had a significantly higher frequency of t (8; 21) and NOS subtypes (*P*=0.049 and 0.005, respectively). In the evaluation of the frequency of t (15; 17), inv (16) and AML with NPM1 mutation, no significant difference was found between two groups (Table 2).

### *EVI1 Expression and Prognostic Marker in AML Patients*

A total of 27 patients including 12 males and 15 females were followed for 48 months. Patients' mean survival was 24.52±3.02 month and their confidence interval was 18.59-30.45 months.

Higher EVI1 expression in AML patients was associated with shorter OS (Figure 1) and concomitant with upper hazard ratio comparison to the patient with low EVI1 expression (Figure 2), but according to Log-Rank test, the difference was not statistically significant (*P*= 0.081). Median OS of the overexpressed group was 10±3.91 months with 2.31-17.68 months confidence interval (Table 3).

## Discussion

EVI1 is a proto-oncogene that controls the proliferation of hematopoietic cells in the early stages of growth.[10] Increasing the expression of EVI1 in hematopoietic progenitor cells can stop myeloid differentiation.[15] Irregular EVI1 expression is caused by a breakpoint near or inside the EVI1 locus at band 3q26.2, which is the prone locus for mutation occurrences such as inv(3)(q21q26.2) and t(3;3)(q21;q26.2). However, EVI1 overexpression occurs in AML patients without chromosome rearrangements in the cited locus.[15]

In this study, we investigated the level of EVI1 expression. Based on the obtained results, EVI1 overexpression was found in 32.95% of AML patients. Previous studies reported EVI1 overexpression in 7.8-21.6% of AML patients.[15, 17, 20] No correlations were found between the level of EVI1expression and patients age, in line with the results of previous studies.[18-21, 28]

The present data did not confirm the correlation between WBC count and low or high expression of EVI1; most of the reports confirmed this observation.[18-21,28] Inconsistent with these results, Ho et al. reported the lower count of WBC in overexpressed EVI1 AML children.[25]

### *The correlation between the level of EVI1 and platelet count was controversial.*

We found a relationship between platelet count and EVI1 expression level; however, it was not statistically significant. This result was in agreement with the study of Groeschel et al. They reported that younger adult patients with high expression of EVI1 have higher platelet counts compared with a patient with low expression, but this correlation was not statistically significant.[20] In a study conducted by Lugthart et al. in the Netherlands, high expression of EVI1 occurred with an increase in platelet count.[19]

Inversely, Barjesteh et al. reported no correlation between platelet count and level of EVI1 expression.[15] Similarly, a recent study by Qin Y.Z. et al. in adult AML patients with intermediate cytogenetic risk receiving

chemotherapy in China revealed a finding the same as that of Barjesteh.[18]

Recently, one report demonstrated the relationship between hemoglobin level and rate of EVI1 expression. These results indicated that overexpression of EVI1 is associated with a significantly lower level of hemoglobin.[18] Our results did not find any associations between over and low expression of EVI1 and hemoglobin level. Such differences may be related to the patients' characteristics or different background.

The correlation between the level of EVI1 expression and FAB classification was controversial, as well.

In our study, there were no meaningful associations between EVI1 overexpression and FAB classification. Similarly, Barjesteh et al., Hass et al., and Qin et al. supported our findings.[15,18,29] On the other hand, Groeschel et al. reported remarkable EVI1 expression in the AML-M4 subgroup.[20] The highest rate of EVI1 expression was reported in AML-M3 by Guangdong and AML-M5 by X He.[21, 22]

We evaluated molecular markers based on the WHO classification in patients under study. The data confirmed the correlation between t (8; 21) and the level of EVI1 expression. AML patients with t (8; 21) revealed a significantly lower expression of EVI1. This result is similar to the result of Groeschel et al. study.[20] X He et al. did not achieve any meaningful correlations between t(8;21) and the rate of EVI1 expression.[21]

In the present study, no correlation was obtained between t(15;17) and the level of EVI1 expression. In this regard, Groeschel et al. and Lugthart et al. reported a lower expression of EVI1 in cases with t(15;17).[19, 20]

Our work did not show any correlation between inv (16) and the level of EVI1 expression. Grosche et al. and Lugthart reported that patients with overexpression of EVI1 do not provide such cytogenetic findings.[19, 20] BV Balgobind et al. did not report overexpression of EVI1 in the pediatric AML patients with favorable molecular risk such as t(8;21), t(15;17), and inv(16).[28]

X He et al. studied EVI1 expression in AML patients with bone marrow transplantation and confirmed a lack of relationship between EVI1 expression level and FLT3 or NPM1 mutation.[21] These findings are consistent with our molecular data; but they are not in agreement with some other studies.[15, 19, 20] Barjesteh, Lugthart, and Groeschel reported that FLT3-ITD and NPM1 mutations had an inverse correlation with overexpression of EVI1. Also, the FLT3-TKD mutation did not show any relation with the EVI1 expression level.[19, 20]

Recently, another study reported no correlation between FLT3-ITD or NPM1 mutations and EVI1 expression rate.[18] The difference between the obtained results can be due to the sample size or different prevalence of mutations in different studies.

The results of the present study demonstrated the decline of patient's OS in EVII overexpressed cases, but it was not statistically significant. Hass et al. and Smol Th et al. declared no relationships between OS and EVI1 overexpression.[29, 30] Nevertheless, other studies have suggested increased EVI1 gene expression in association with OS decline.[15,18-20,23] It seems that these huge differences are due to the number of patients, patients' characteristics, and the definition of the cut-off value.

## Conclusion

Based on the results of this study, EVI1 expression increases in AML patients compared to healthy subjects. Such an increase did not show any relation with the age and gender of the patients and AML FAB classification. Overexpression was more common in patients with higher PLT count. Survival was not significantly correlated with the level of EVI1 expression. It seems that EVI1 expression is not applicable as a prognostic factor alone. Other factors such as monosomy 7 and 11q23 rearrangements may lead to less survival in patients with increased expression of EVI1.

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## Conflict of Interest

None declared.

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