




## Expression of DR4, DR5, FAS, Caspase-8 and, DDIAS Genes in AML Patients

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### Abstract

**Background:** Acute myeloid leukemia (AML) is the most common acute leukemia in adults and accompanies a worse survival. In this study, gene expression levels of 5 key players of apoptosis, including DR4, DR5, FAS, caspase 8, and DNA damage-induced apoptosis suppressor (DDIAS), have been evaluated in AML patients compared with controls, aiming to evaluate their possible role and prognostic impact.

**Methods:** This cross-sectional study was performed in the Cancer Molecular Pathology Research Center, Mashhad University of Medical Sciences. A total of 30 newly diagnosed AML cases as well as 30 healthy controls enrolled in the study. Real-time polymerase chain reaction was used to evaluate the expressions of DR4, DR5, FAS, DDIAS, and caspase 8 genes in cases and controls. Other necessary data, including cytogenetic findings, mutations, French-American-British (FAB) classification, and survival, were retrieved from hospital records and by direct contact with patients. Statistical analysis was done by SPSS software. When appropriate, the Mann-Whitney U, Pearson's correlation, and the t tests were utilized. Overall survival (OS) was estimated using the Kaplan-Meier method.

**Results:** The expression of all evaluated genes, including DDIAS ( $0.89 \pm 0.20$ ), DR4 ( $0.67 \pm 0.24$ ), DR5 ( $0.72 \pm 0.24$ ), FAS ( $0.70 \pm 0.25$ ), and Caspase 8 ( $0.77 \pm 0.20$ ) were significantly decreased in AML patients compared with the controls ( $P < 0.001$ ). Patients with the t (16;16) or inv (16) expressed significantly higher amounts of the FAS gene and those with FLT3 mutation exhibited lower expression of caspase 8. Expression of the evaluated genes showed no significant effect on survival.

**Conclusion:** The expression of DR4, DR5, FAS, and caspase 8 seems to be decreased in AML. Lower expression of these molecules may aid AML cells in avoiding apoptosis because they are involved in the initiation of apoptosis, making them potential targets for treatment.

**Keywords:** Acute Myeloid Leukemia, Apoptosis, DDIAS, DR4, DR5, FAS, Caspase 8

**Conflicts of Interest:** None declared

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### Introduction

Acute myeloid leukemia (AML) is the most common type of acute leukemia in adults with a median age at di-

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#### ↑What is "already known" in this topic:

Apoptosis inhibition is a fundamental aspect of carcinogenesis that has been achieved by different mechanisms in the vast majority of cancers. Researching various facets of the apoptotic process is essential to discover ways to overcome this inhibition and promote apoptosis in cancer cells. Conflicting data have been reported about the expression of FAS, DR4, and DR5 in AML; and to the best of our knowledge, there are no data regarding the expression of DDIAS in AML patients.

#### →What this article adds:

In the present study, gene expression levels of some key players of the apoptotic pathway, including FAS, DR4 and DR5, caspase 8, and DDIAS, an inhibitor of apoptosis, have been evaluated in AML patients and all of them showed lower expression in AML patients compared with controls. As FAS, DR4, and DR5, caspase 8 are involved in the initiation of apoptotic pathways, and their lower expression could help neoplastic cells evade apoptosis and may be used as therapeutic targets.

agnosis of 67 years (1). Despite various treatment protocols, the 5-year survival for the majority of patients is low (2). Some cytogenetic markers and genetic mutations are significantly associated with risk stratification and prognosis and now are included in the World Health Organization classification of AML. A better understanding of the genetic and molecular basis of AML may lead to improve treatment outcomes for patients. Evasion of programmed cell death (apoptosis) is a key step in cancer pathogenesis (3). There are 2 main apoptotic pathways: extrinsic and intrinsic. The extrinsic pathway of apoptosis is mediated by a group of cell surface receptors, including FAS (CD95), tumor necrosis factor-related apoptosis-inducing ligand receptor-1 (TRAIL-R1/DR4), and receptor 2 (TRAIL-R2/DR5). After stimulation by their specific ligands, such as FASL and TRAIL, these receptors activate an intracytoplasmic signaling pathway that through activation of caspase 8 and other caspases leads to apoptotic cell death (4). TNF-related apoptosis-inducing ligand (TRAIL) is a propitious therapeutic potential, as cancer cells are more sensitive to TRAIL-mediated apoptosis than healthy cells, regardless of TP53 activation. Recently, several studies have indicated that the antiapoptotic protein DNA damage induced apoptosis suppressor (DDIAS) overexpression contributed to the progression and chemoresistance of human malignancies. DDIAS protects cancer cells from apoptosis in different ways (5), including prevention of death-inducing signaling complex (DISC) formation as well as induction of caspase 8 ubiquitination (6). Although DDIAS has shown surprising functions in apoptosis dysregulation in some human malignancies and has been involved in the induction of chemotherapy resistance, to the best of our knowledge, its expression has not been evaluated in hematological malignancies.

This study was designed to evaluate the expression of apoptosis pathway genes (FAS, DR4, DR5, caspase 8, and DDIAS) in AML patients and their correlation with prognostic factors and survival.

## Methods

### Participants

For this cross-sectional, bone marrow (BM) and peripheral blood (PB) samples of 30 newly diagnosed AML patients who had been admitted to the Hematology and molecular laboratory of Ghaem University Hospital, Mashhad University of Medical Sciences (MUMS), were enrolled. Only patients with more than 80% blast cells in their specimens were included. Demographic, clinical, and laboratory data of patients, including age, gender, complete blood count (CBC) findings, FAB classification, karyotyping, gene mutation, and survival rate were ex-

tracted from hospital electronic files. In addition, 30 age and sex-matched controls were involved and their peripheral blood samples were used for the study. The study protocol was approved by the ethics committee of the MUMS (committee code: IR.MUMS.MEDICAL.REC.1397.475).

### RNA Extraction and cDNA Synthesis

Total RNA was extracted from BM and PB mononuclear cells using Trizol Reagent (Invitrogen) according to the manufacturer's instructions and purified by DNase-I (Thermo, USA). Also, 1 µg of total RNA was used for cDNAs synthesis using Revert Aid H Minus First Strand cDNA Synthesis Kit (No.: K1621; Thermo Co). The quality of synthesized cDNA was determined by NanoDrop spectrophotometer (Thermo Scientific NanoDrop2000).

### Reverse Transcriptase Quantitative Polymerase Chain Reaction

The primer pairs were designed for each gene as shown in Table 1. To normalize the expression data, GAPDH was used as the housekeeping gene. Primer-BLAST was done to confirm the specificity of the primers (www.blast.ncbi.nlm.nih.gov).

Real-time polymerase chain reactions were performed by the Applied Bio-system Step One Plus Real-Time Polymerase Chain Reaction (PCR) Systems (Applied Biosystems), using the cyber green method. The reaction mix was composed of 10 µL SYBR Green Master Mix (2X) (K0221, Fermentas), 0.25 µL of each primer (10 pmol/µL), 2 µL cDNA, and 7.5 µL of nuclease-free water in a total volume of 20 µL. The tests were done twice. Two negative controls were also involved in each assay. The thermal cycle conditions of the study for each studied gene were initial denaturation: 10 min at 95°C followed by 40 cycles; denaturation: 95°C for 30 sec; annealing: 63°C for DDIAS, 57°C for FAS, 60°C for DR4, 59°C for DR5, and 55°C for caspase 8 for 40 sec; extension: 72°C for 30 sec. Then, a melt curve analysis was also done. The amount of gene expression was calculated by the  $2^{-\Delta\Delta Ct}$  method.

### Statistical Analysis

Statistical analysis was performed by SPSS Version 26 software. To compare continuous variables, a t test or a Mann-Whitney U test was performed. The correlation between the expression of different genes was done by the Pearson correlation test. The overall survival (OS) was estimated using the Kaplan- Meier method. The effect of different factors on survival has been evaluated with the log-rank test.  $P < 0.05$  were considered statistically signif-

Table 1. q-PCR primer sequences

Name	Forward primer	Reverse primer	Product Size
Fas	5'-ATGCACACTCTGCGATGAAG-3'	5'-CAGTGTTCACAGCCAGGAGA-3'	104 pb*
Caspase-8	5'-CTGCTGGGGATGGCCACTGTG-3'	5'-TCGCCTCGAGGACATCGCTCTC-3'	113 bp*
DR4	5'-TCCAGCAAATGGTGCTGAC-3'	5'-GAGTCAAAGGGCACGATGTT-3'	78 bp*
DR5	5'-CCAGCAAATGAAGGTGATCC-3'	5'-GCACCAAGTCTGCAAAGTCA-3'	67 bp*
DDIAS	5'-GTATCCCTCCA CTTCTTG-3'	5'-TGCTGATGCCATTTACTGAT-3'	186 bp*
GAPDH	5'-TGCACCACCAACTGCTTAG-3'	5'-GGATGCAGGGATGATGTT-3'	220 bp*

\* bp: base pair

icant.

## Results

A total of 30 patients, including 19 (64%) men and 11 (36%) women with a mean (SD) age of  $64.57 \pm 13.8$  years, ranging between 36 to 88 years, were enrolled in this study. The control group included 30 participants with same-sex distribution and mean (SD) age of  $62.71 \pm 12.9$  years, ranging between 28 to 83 years. Table 2 shows the demographic and laboratory findings of patients and controls.

The expression of all evaluated genes, including DDIAS ( $0.89 \pm 0.20$ ), DR4 ( $0.67 \pm 0.24$ ), DR5 ( $0.72 \pm 0.24$ ), FAS ( $0.70 \pm 0.25$ ), and Caspase 8 ( $0.77 \pm 0.20$ ), were significantly decreased compared with controls ( $P < 0.001$ ).

The association between expression of genes and cytogenetic changes as well as gene mutations are summarized in Table 3. Significantly lower expression of DR5 was seen in patients with NPM1 and RUNX1 mutations as well as patients with t(8;21). In addition, patients with t(8;21) also showed significantly lower expressions of DR4. Patients with t(16;16) or inv(16) expressed significantly higher amounts of the FAS gene and those with FLT3 mutation exhibited lower expression of caspase 8.

Expressions of these genes did not show any significant association with FAB classification and CBC findings or the sex and age of the patients.

In addition, a positive correlation was identified between the expression of FAS with DR5 and Caspase 8 ( $P = 0.019$ ;  $r = 0.425$ ) and ( $P = 0.016$ ;  $r = 0.435$ ), respectively). Also, there was a positive correlation between the expression of DR5 with DR4 ( $P < 0.001$ ;  $r = 0.648$ ).

The downregulation of the examined genes did not significantly affect the survival of patients, according to Kaplan-Meier analysis (Figure 1).

## Discussion

Apoptosis inhibition is a fundamental aspect of carcinogenesis that has been achieved by different mechanisms in the vast majority of cancers. In order to uncover ways to get around this inhibition and make cancer cells more susceptible to apoptosis, it is crucial to research several aspects of the apoptotic pathway. In the present study, expression levels of some key players of the apoptotic pathway, including FAS, DR4, DR5 (death receptors), caspase 8 (an important effector molecule in the process), and DDIAS (an inhibitor of apoptosis), have been evaluated in AML patients and controls using real-time PCR method.

The expression of all evaluated death receptors, including FAS, DR4, and DR5, were significantly lower in AML patients compared to the controls in this study. Lower expression of these molecules, which are involved in the commencement of the apoptotic pathway, aids cancerous cells in avoiding apoptosis. In line with our results, Prodzik et al, using flowcytometry, reported a significantly lower expression of FAS and DR4 in AML patients compared to controls (3). As the mentioned study evaluated the protein expression, it also confirms that gene expression levels of FAS and DR4 show a good correlation with the protein expression levels. Furthermore, it has been shown that AML cells are resistant to FAS-mediated apoptosis (7). Although the mechanism of this resistance was not investigated, the authors proposed a decrease in FAS expression, soluble FAS secretion, or a change in

Table 2. Laboratory and clinical findings in patients and control groups

Variable	Patients	Control	P-value
Age (years)	$64.57 \pm 13.8$	$62.71 \pm 12.9$	0.591
gender (M/F)	19 / 11	19 / 11	1.000
RBC ( $\times 10^6/\mu\text{l}$ )	$2.99 \pm 0.82$	$3.9 \pm 0.22$	<0.001
WBC ( $\times 10^3/\mu\text{l}$ )	$31.7 \pm 29.62$	$7.3 \pm 1.82$	<0.001
Hb (gr/dl)	$8.27 \pm 2.59$	$13.1 \pm 0.6$	<0.001
HCT (%)	$25.54 \pm 6.95$	$40.5 \pm 2.1$	<0.001
PLT ( $\times 10^3/\mu\text{l}$ )	$74.3 \pm 45.1$	$295.8 \pm 38.2$	<0.001

M: male/ F: female/ RBC: red blood cell/ WBC: white blood cell/ PLT: platelet/ Hb: hemoglobin/ HCT: hematocrit/ NS: not significant.

Table 3. Correlation of DR4, DR5, FAS, Caspase-8 and DDIAS Gene Expressions with cytogenetic changes and gene mutations in patients with AML

AML subtype		DR4 Expression		DR5 Expression		FAS Expression		Caspase8 Expression		DDIAS Expression	
		Mean $\pm$ SD	P. Value	Mean $\pm$ SD	P. Value	Mean $\pm$ SD	P. Value	Mean $\pm$ SD	P. Value	Mean $\pm$ SD	P. Value
t(8;21)	Absent	0.75 $\pm$ 0.25	0.004	0.78 $\pm$ 0.23	0.024	0.73 $\pm$ 0.28	0.344	0.80 $\pm$ 0.21	0.202	0.86 $\pm$ 0.12	0.428
	Present	0.48 $\pm$ 0.09		0.55 $\pm$ 0.20		0.64 $\pm$ 0.17		0.69 $\pm$ 0.18		0.84 $\pm$ 0.12	
t(15;17)	Absent	0.69 $\pm$ 0.29	0.720	0.72 $\pm$ 0.23	0.967	0.74 $\pm$ 0.25	0.363	0.78 $\pm$ 0.22	0.902	0.90 $\pm$ 0.00	0.136
	Present	0.66 $\pm$ 0.22		0.71 $\pm$ 0.25		0.68 $\pm$ 0.26		0.76 $\pm$ 0.20		0.85 $\pm$ 0.12	
t(16;16) or inv16	Absent	0.64 $\pm$ 0.22	0.320	0.71 $\pm$ 0.24	0.662	0.65 $\pm$ 0.26	0.040	0.75 $\pm$ 0.21	0.504	0.83 $\pm$ 0.09	0.298
	Present	0.78 $\pm$ 0.31		0.74 $\pm$ 0.25		0.85 $\pm$ 0.18		0.81 $\pm$ 0.19		0.88 $\pm$ 0.14	
t(9;11)	Absent	0.69 $\pm$ 0.26	0.416	0.74 $\pm$ 0.22	0.385	0.73 $\pm$ 0.26	0.188	0.78 $\pm$ 0.21	0.787	0.98 $\pm$ 0.14	0.914
	Present	0.58 $\pm$ 0.19		0.60 $\pm$ 0.31		0.58 $\pm$ 0.18		0.74 $\pm$ 0.18		1.00 $\pm$ 0.0	
t(9;22)	Absent	0.70 $\pm$ 0.25	0.139	0.75 $\pm$ 0.23	0.094	0.71 $\pm$ 0.27	0.498	0.78 $\pm$ 0.21	0.359	0.98 $\pm$ 0.01	0.930
	Present	0.50 $\pm$ 0.08		0.50 $\pm$ 0.24		0.65 $\pm$ 0.10		0.67 $\pm$ 0.13		1.00 $\pm$ 0.00	
Normal Karyotype	Absent	0.67 $\pm$ 0.25	0.556	0.70 $\pm$ 0.24	0.414	0.71 $\pm$ 0.25	0.556	0.77 $\pm$ 0.20	0.901	0.99 $\pm$ 0.13	0.372
	Present	0.75 $\pm$ 0.21		0.90 $\pm$ 0.00		0.55 $\pm$ 0.35		0.75 $\pm$ 0.35		0.90 $\pm$ 0.14	
NPM1 Mutation	Absent	0.73 $\pm$ 0.27	0.171	0.79 $\pm$ 0.20	0.037	0.71 $\pm$ 0.25	0.553	0.76 $\pm$ 0.22	0.800	0.98 $\pm$ 0.16	0.832
	Present	0.58 $\pm$ 0.18		0.59 $\pm$ 0.26		0.70 $\pm$ 0.26		0.78 $\pm$ 0.17		1.00 $\pm$ 0.00	
FLT3 Mutation	Absent	0.73 $\pm$ 0.26	0.198	0.73 $\pm$ 0.27	0.621	0.77 $\pm$ 0.27	0.113	0.85 $\pm$ 0.17	0.025	0.98 $\pm$ 0.14	0.805
	Present	0.60 $\pm$ 0.21		0.70 $\pm$ 0.20		0.62 $\pm$ 0.21		0.67 $\pm$ 0.21		1.00 $\pm$ 0.00	
RUNX1 Mutation	Absent	0.69 $\pm$ 0.25	0.200	0.75 $\pm$ 0.22	0.072	0.71 $\pm$ 0.26	0.695	0.78 $\pm$ 0.21	0.387	0.98 $\pm$ 0.14	0.948
	Present	0.50 $\pm$ 0.10		0.43 $\pm$ 0.25		0.67 $\pm$ 0.12		0.67 $\pm$ 0.15		1.00 $\pm$ 0.00	

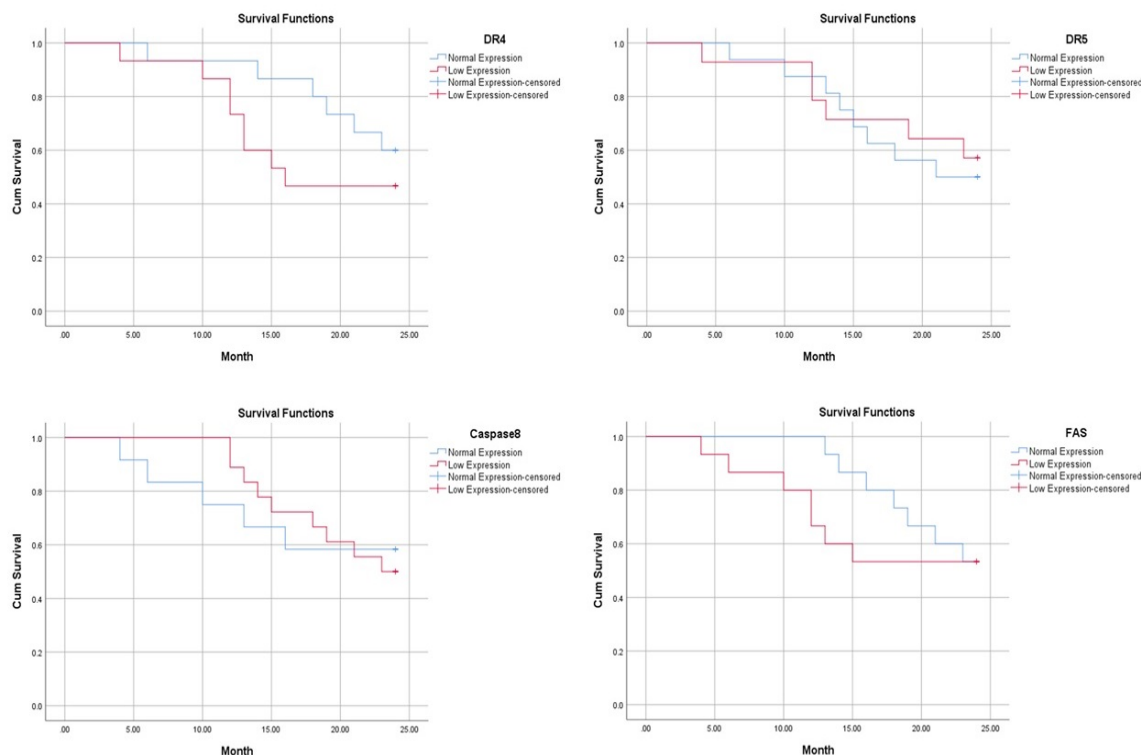


Figure 1. Kaplan-Meier Plot Between Normal and Low Expression of DR4, DR5, Caspase 8 and Fas. No correlation was found between down regulation of these genes and survival ( $P < 0.05$ ).

intracellular apoptotic resistance as possible mechanisms, which further confirms our results. In a study by Tahir et al, evaluating the activity of the TRAIL receptor agonistic fusion protein eftozanermin alfa (eftoza) in combination with venetoclax in AML preclinical models and patients, Researchers discovered that Eftoza action needed the expression of DR4/DR5 on the cell surface and that combining Eftoza and venetoclax, which stimulates both extrinsic and intrinsic apoptosis-signaling pathways, may improve clinical outcomes. These findings further emphasize the importance of the expression of death receptors and their evaluation in the pathogenesis and treatment of patients with AML (8).

In contrast to our findings, Lin et al reported an increase in the expression of the FAS gene in AML cases compared to controls (9). These differences may be related to heterogeneity of patients and control groups, different ethnicity of patients, or different sample sizes.

Additionally, Prodzik et al found that AML patients with favorable cytogenetic findings exhibited higher percentages of cells positive for FAS, DR4, or DR5 (3). This may have prone cells to apoptotic death, leading to a better prognosis. In line with their report, we found that AML patients with inv (16) or t (16;16), which indicates a favorable prognostic group, have significantly higher FAS gene expression than others. However, other patients in groups with favorable prognoses did not exhibit this, and it is possible that other mechanisms contributed to their good behavior. Prodzik et al also reported that cases with higher DR4-positive cells showed better survival rates (3). We did not find any correlations between survival and expression of these genes. In line with the present study,

Min et al's use of flow cytometry and immunohistochemistry demonstrated that overall survival did not differ substantially between AML cases that were positive for FAS or DR4 or those that were not. However, they found that relapse-free survival was prolonged in FAS-positive cases compared to FAS negative ones (4). In addition, Chen et al, evaluating the prognostic value of FAS mRNA in various malignancies using online platforms, reported that higher expression of FAS was related to a poorer OS in AML patients (Hazard ratio, 1.57;  $P = 0.04$ ) (10).

Different methods used in these studies may explain the different findings. Further studies with higher sample sizes are needed to elucidate the prognostic impacts of the expression of these genes in AML patients.

Following activation of death receptors by their ligands, they directly attach to FADD (FAS-associated death domain), which recruits procaspase 8 as the main effector of the apoptotic pathway, to make the death-inducing signaling complex (DISC) (11, 12). In the present study, the expression of caspase 8 was significantly lower in AML patients compared to controls, which may indicate another defense mechanism against apoptosis in AML. The effect of changes in caspase 8 on the behavior of AML cells has been investigated by others. Evaluating blasts of 25 AML cases, Riccioni et al, reported that a low caspase 8 level was associated with resistance to triterpenoid CDDO-Imidazolide (13). Ming Li et al demonstrated that AML patients with caspase 8 Q482H mutation were more resistant to chemotherapy and this mutation leads to abolished TRAIL-mediated apoptosis (14). Patients with FLT3 mutation showed lower expression of caspase 8 than other patients in the present study. As the FLT3 mutation im-

plies a worse prognostic group, this may indicate the role of a decrease in caspase 8 in this behavior. Anyway, further studies are needed to explore the role of caspase 8 in the pathogenesis and prognosis of AML cases.

DDIAS is a novel anti-apoptotic gene that is broadly expressed in some cancers such as lung and hepatocellular carcinoma and is associated with poor prognosis (5, 6, 15). According to reports, DDIAS prevents TRAIL-mediated apoptosis by preventing the formation of DISKS and caspase 8 ubiquitination (6). Nan Liu et al showed that DDIAS overexpression is associated with poor survival and progression of non-small lung cancer (16). DDIAS showed lower expressions in AML patients in the present study. Regarding the antiapoptotic role of DDIAS, one may expect up-regulation of this gene during carcinogenesis. Nevertheless, as the various mechanisms used in different cancers for the same reason, DDIAS may not be involved in the apoptotic inhibition in AML blasts and other ways may have been employed. Because of the small sample size of our study and the lack of comparable studies reporting the evaluation of DDIAS in AML, further studies with larger sample sizes are required to assess the expression and involvement of DDIAS in the pathogenesis of AML.

### Conclusion

In the present study, gene expression levels of some key players of the apoptotic pathway, including FAS, DR4 and DR5, caspase 8, and DDIAS, an inhibitor of apoptosis, have been evaluated in AML patients and all of them showed lower expressions in AML patients compared to controls. As FAS, DR4, DR5, and caspase 8 are involved in the initiation of apoptotic pathways, their lower expressions could help neoplastic cells evade apoptosis. The level of expression of these genes showed no effect on survival. However, patients with inv (16) or t (16;16), a favorable prognostic group, showed upper expression of FAS and patients with FLT3 mutation, a lower prognostic group, showed lower expressions of caspase 8, which may indicate a prognostic impact for expression levels of these molecules. Further studies, with larger sample sizes as well as studies that evaluate the functional effects of these changes on cellular pathways, are needed to elucidate the role and prognostic impact of these molecules in AML pathogenesis.

### Ethical Approval

This study was performed in line with the principles of the Declaration of Helsinki. The study protocol was approved by the Research Ethics Committee at Mashhad University of Medical Sciences (IR.MUMS.MEDICAL.REC.1397.475).

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### Authors Contributions

J.N., S.B., H.A., and M.S. conceived and designed the study. J.N., M.S., H.A., M.H.S., and A.A. performed the experimental work. J.N., S.B., A.A., Z.K., and M.S. participated in extracting the needed data. J.N., S.B., Z.K., and A.A. did the statistical analysis and prepared the primary draft. The final version was examined and approved by all authors who participated in editing the initial draft.

### Conflict of Interests

The authors declare that they have no competing interests.

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