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Involvement Value of FLT-3, c-Myc, STAT3, p27, and HOTAIR Gene Expression in Acute Myeloid Leukemia Patients: A Molecular Perspective to a Novel Leukemogenesis Mechanism

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ABSTRACT

Background: The identification of long non-coding RNAs (lncRNAs) in the pathogenesis of acute myeloid leukemia (AML) has marked a new era in the molecular understating of the disease. This study investigated the correlation between the changes in the expression of lncRNAs, including HOTAIR, PVT-1, and CRNDE, and the alteration in the expression profile of FLT-3, c-Myc, STAT3, STAT5, and p27 in AML patients.

Materials and Methods: Blood samples were collected from forty-one newly diagnosed AML patients and ten healthy individuals to evaluate the expression levels of the study genes using qRT-PCR analysis. The probable correlation between the gene expressions was determined using Pearson's correlation test.

Results: The results showed that while there was a significant elevation in the expression of FLT3, c-Myc, STAT3, and HOTAIR, p27 expression remarkably diminished in AML patients compared to the control group. Also, a correlation was found between the expression of FLT-3 and p27 and the expression of HOTAIR and STAT3. It was assumed that FLT-3 had a role in increasing the proliferative and survival capacity of AML cells, at least partly, through c-Myc-mediated suppression of p27. Moreover, lncRNA HOTAIR showed to be involved in leukemia proliferation assumably by enhancing the expression of STAT3.

Conclusion: Overall, the results of gene profile analysis suggested that studying the expression of HOTAIR, FLT-3, c-Myc, STAT3, and p27 could be helpful to AML patients, and each of these genes could be a valuable target for pharmaceutic intervention.

Keywords: Acute myeloid leukemia; LncRNAs; Gene expression; HOTAIR; FLT-3

INTRODUCTION

Acute myeloid leukemia (AML) is an infrequent, highly mortal malignancy affecting individuals in two age groups; early childhood and adulthood[1]. It is a heterogeneous disease developed due to the aberrancies in the proliferation and differentiation of

the clonal population of myeloid stem cells. The genomic analysis revealed the incidence of 50% to 80% of acquired genetic disorders, such as point mutations, deletions, and translocation in cases of AML[2, 3]. Although many efforts have been made to describe the precise pathogenesis of AML, still, numerous questions regarding the primary mechanism involved in leukemogenesis remained unanswered.

As the number of genes linked to the pathogenesis of AML is growing, the identification of the essential genes associated with the early stage of leukemogenesis seems impossible. However, the identification of a group of regulatory RNAs - long non-coding RNAs - has shed another light on the pathogenesis of not only AML but many human cancers. With the advent of RNA sequencing technologies, it has been indicated that lncRNAs are involved in post-translational modification regulation of a wide range of genes and proteins, and their regulation might have critical roles in the development of leukemia[4,5]. lncRNAs have both tumor suppressive and oncogenic functions. The association of several lncRNAs with the development of AML has been well-established. It has been reported that PVT-1 increases AML development by interacting with c-Myc, thereby increasing the cells' proliferation[6]. ANRIL is another oncogenic lncRNA that participates in leukemogenesis through suppressing the INK4b-ARF-INK4a complex, a group of transcription factors that are necessary for the differentiation of both myeloid and lymphoid lineage7. Guo et al. indicated that high expression of H19 was required for the development of AML, as this lncRNA alters the expression levels of RB[8] . IRAIN and MEG3 are tumor-suppressive lncRNAs that their expression was dramatically decreased during AML. The downregulation of IRAIN and MEG3 is associated with the activation of the IGF1R/PI3K signaling pathway[9,10] and suppression of p53 [11,12], respectively. Given the significant role of lncRNAs in the regulation of the genes that participated in AML development, this study aimed to investigate the correlation between the changes in the expression of some lncRNAs, including HOTAIR, PVT-1, and CRNDE, and the alteration in the expression profile of FLT-3, c-Myc, STAT3, STAT5, and p27.

MATERIALS AND METHODS

Patients sample collection

The peripheral blood samples were collected from forty-one newly diagnosed AML patients and ten healthy volunteers. The Research Ethics Committee approved the research protocol at the Shahid Beheshti University of Medical Sciences (ID number: IR.SBMU.RETECH.REC.1397.1012), and all participants signed an informed consent document in accordance with the Helsinki declaration. Table 1 presents the clinical characteristics of the patients. The samples of AML-M3 were excluded from the study. Patients had an average age of 54/6 years, with the youngest aging 17 and the oldest aging 80 years. Moreover, 13 out of 41 patients (31.7%) were women, and 28 out of 41 (68.2%) were men.

RNA purification and preparation of cDNA

After harvesting mononuclear cells (MNCs) from the samples using ficol, the total RNA was extracted from the cells by a High Pure RNA isolation kit (Qiagen, Germany). The quality and the quantity of extracted RNA were detected by Nanodrop ND-1000 (Optical Density (OD) 260/280nmratio> 1.8). One microgram of the extracted RNAs was subjected to a cDNA synthesis kit (Thermo scientific - USA) to produce the cDNAs required for further analysis.

Real-time quantitative PCR

The sense and antisense primer of the desired genes were designed in Gene Runner. Then, the changes in gene expression levels were evaluated using SYBR green-based real-time quantitative polymerase chain reaction (qRT-PCR) (Rotor-Gene 6000, Qiagen, Germany). In a total volume of 15 µL, we mixed 2 µL of cDNA, 1 µL of sense and antisense primers, 7.5 µL of master Mix, and 4.5 µL of water. The mixture was then placed in a thermal cycler. ABL was amplified as housekeeping genes. All tests were done in triplicate, and fold changes in the expressions of each mRNA were calculated using $2^{-\Delta\Delta Ct}$.

Statistical analysis

The statistical analysis was performed in SPSS (Ver. 21.0) and GraphPad Prism. Independent student I or Mann-Whitney U tests were conducted to compare patients and the control group. Pearson's correlation

test was used to study the possible correlation between the indicated genes. A probability value of less than 0.05 was considered statistically significant.

RESULTS

The expression level of FLT-3, c-Myc, and PIM1 in AML samples

FLT-3 is an important receptor expressed on the surface of the leukemic cells that regulates the AML [13, 14]; however, it is not yet clear whether the up-regulation of this receptor is detectable at the time of the diagnosis or relapse. To assess the expression level of FLT3 and its downstream target c-Myc in AML and explore its plausible contributory role in leukemia development, peripheral blood of 41 AML patients was detected using qRT-PCR. The results showed an increased expression of FLT-3 in the patient group compared to the control group (Figure 1). The previous studies reported that the expression of c-Myc and PIM1 frequently increased in AML patients. However, when the expression of these genes was evaluated in the study group, it was found that, unlike c-Myc, which its expression significantly increased, the alteration in the expression of PIM1 was not statistically significant (Figure 1). Based on the results, it was of particular interest to evaluate whether the alteration in the expression pattern of these genes has any association with age, gender, and the percentage of blasts. A similar expression pattern was found both in male and female groups and in all age categories in which the samples were divided. The same result was obtained when the mRNA levels of these genes were compared with blast percentages (Table 2).

Correlation between FLT-3, c-Myc, and PIM1 expression levels in AML patients

With the significant increase in the expression of FLT-3 and c-Myc in AML patients, statistical correlation analysis was used to evaluate any correlation between the expression levels of the genes in the patients' group. The results showed that although the expression of FLT-3 and c-Myc had a positive correlation ($r = 0.296$), it was not statistically significant (Figure 2). Moreover, no correlation was found between FLT-3 and PIM1 and c-Myc and PIM1 (Figure 2).

The expression level of STAT3 and STAT5 in AML samples

The expression of STAT3 and STAT5 in AML patients and the control group was evaluated. The results showed that among 41 patients, 24 had up-regulated STAT3, and 38 had up-regulated STAT5. However, compared to the control group, the expression of STAT3 was significant in the AML group. (Figure 3a). Then, the association between alteration in the expression pattern of STAT3 with age, gender, and the percentage of blasts was investigated: no correlation was found (Table 3).

The expression level of p27 in AML samples

Since the STAT signaling pathway regulates cell proliferation, we aimed to investigate the expression of p27 in AML patients. It was found that among 41 patients, 24 had downregulated p27. Furthermore, compared to the control group, the downregulation in p27 was statistically significant (Figure 3b). Also, the expression of p27 did not correlate with age, gender, and blast percentage (Table 2). The Pearson correlation test was used to evaluate whether there was a significant correlation between the expression levels of STAT3 and p27 in the patient's group. The test results indicated no significant correlation between STAT3 and p27 in patients ($r = 0.093$ $P \leq 0.591$). Additionally, the correlation between p27 and FLT-3 and p27 and c-Myc was evaluated. The results showed a significant correlation between p27 and FLT-3 ($r = 0.406$, $P < 0.014$) (Figure 4).

The expression level of HOTAIR, PVT1, and CRNDE in AML samples

The results of qRT-PCR showed that while the expression of HOTAIR and PVT1 increased in most patients, the expression of CRNDE decreased. The observations indicated that only the overexpression of HOTAIR was significant in patients compared to PVT1 and CRNDE (Figure 5). Given this, it was of particular interest to evaluate whether the alteration in the expression pattern of HOTAIR has any association with age, gender, and the percentage of blasts. A similar expression pattern was found x in male and female groups and all age categories (Table 3). The same result was obtained when comparing

the mRNA level of these genes with the percentage of the blasts in Table 4.

Correlation between HOTAIR, FLT-3, c-Myc, STAT3, and p27 expression in AML patients

Having established a significant increase in the expression of HOTAIR in AML patients, the statistical correlation analysis method was used to evaluate any significant correlation between the expression levels of the HOTAIR, FLT-3, c-Myc, STAT3, and p27 in the study group. Among all genes, HOTAIR had significant correlation with STAT3 ($r = 0.495$, $P \leq 0.002$) (Figure. 6).

Table 1. The Clinical Characteristics of De Novo Acute Myeloid Leukemia Patients

Patients	FAB	WBC × 10^3	PLT × 10^3	Hb (g/dl)	Blast (%)	Flow cytometry
1	AML-M5b	46	115	11.1	37	34,14,64,13,33,DR,11b,4dim,45+
2	AML-M2	32	91	10.8	60	34,13,33,DR,117+
3	AML-M1	22	127	11.4	28	34,13,33,DR,117+
4	AML-M1	67.4	77	12.1	60	34,13,33,DR,117+
5	AML-M1	3.3	67	11	42	34,13,33,DR,117+
6	AML-M2	2	100	7.5	30	34,13,33,DR,117+
7	AML-M1	1.4	77	9	40	34,13,33,DR,117+
8	AML-M2	1.7	80	10	35	34,13,33,DR,117+
9	AML-M4	29.8	73	9.3	70	64,14,34,13,33,DR,117+
10	AML-M1	112	47	9	90	34,13,33,DR,117+
11	AML-M0	8.2	81	9.9	82	34,10,DR,45dim
12	AML-M2	22	100	9.2	57	34,13,33,DR,117+
13	AML-M1	18	101	11.1	52	34,13,33,DR,117+
14	AML-M2	9.7	81	9.3	71	34,13,64,33,DR,117+
15	AML-M1	3.1	72	10.2	30	34,13,33,DR,117+
16	AML-M1	29	62	9	60	34,13,33,DR,117+
17	AMLM4	62	92	10	45	64,14,34,13,33,DR,117+
18	AML-M2	38	70	8.1	75	34,13,64,33,DR,117+
19	AML-M2	21	68	7.5	32	34,13,64,,33,DR,117+
20	AML-M2	110	51	6.7	80	34,13,33,DR,117+
21	AML-M2	64	94	10.8	47	34,13,33,DR,117+
22	AML-M1	56	111	8.4	92	34,13,33,DR,117+
23	AML-M1	50	94	9	88	34,13,33,DR,117+
24	AML-M2	60	51	9.2	52	34,13,33,DR,117+
25	AML-M1	52	65	8.2	49	34,13,33,DR,117+
26	AML-M2	3.5	100	9.1	31	34,13,33,DR,117+
27	AML-M1	4	82	8.2	35	34,13,33,DR,117+
28	AML-M2	340	92	9.1	80	34,13,33,DR,117+
29	AML-M1	15	111	8.1	30	34,13,33,DR,117+
30	AML-M1	4.5	82	9	80	34,13,33,DR,117+
31	AML-M2	210	70	7.1	75	34,13,33,DR,117+
32	AML-M2	280	89	6.1	78	34,13,33,DR,117+
33	AML-M2	20	100	11.1	34	34,13,33,DR,117+
34	AML-M2	16	112	11.5	37	34,13,33,DR,117+
35	AML-M2	212	92	7.5	73	34,13,33,DR,117+
36	AML-M5a	300	60	9.7	85	34,14,64,13,33,DR,45+
37	AML-M4	20	85	8.2	40	64,14,34,13,33,DR,117+
38	AML-M2	25	111	8.7	50	34,13,33,DR,117+
39	AML-M5b	27.5	120	9.1	32	34,14,64,13,33,DR,11b,4dim,45+
40	AML-M5a	312	85	10.2	81	34,14,64,13,33,DR,45+
41	AML-M2	23	95	8	45	34,13,33,DR,117+

Table 2. The evaluation of the gene expression of FLT-3 and c-Myc in AML patients [a,b]

Clinical variables	Total Patients, 41 (100)	Evaluable patients			Evaluable patients		
		FLT-3 (↑)	FLT-3 (↓)	*P*	c-Myc (↑)	c-Myc (↓)	*P*
Age (year)	54.6, 17-80			0.25			0.41
n < 60	22 (53.65)	15 (68.18)	7 (31.81)		13 (59.09)	9 (40.90)	
n ≥ 60	19 (46.35)	4 (21.05)	12 (63.15)		11 (57.89)	8 (42.1)	
Sex				0.7			0.12
Female	13 (31.7)	10 (76.92)	3 (23.07)		10 (76.92)	3 (23.07)	
Male	28 (68.2)	18 (64.28)	10 (35.73)		18 (64.28)	10 (35.71)	
Blast percentage				0.46			0.17
n < 40	12 (29.26)	9 (75)	4 (15)		5 (41.66)	7 (57.33)	
n ≥ 40	30 (70.74)	19 (63.33)	9 (30)		19 (63.33)	10 (33.33)	

Abbreviation: AML, acute myeloid leukemia
aValues are expressed as No. (%) or median, range
bStatistically significant values: P < 0.05

Table 3. The evaluation of the gene expression of STAT3 and p27 in AML patients [a,b]

Clinical variables	Total Patients, 41 (100)	Evaluable patients			Evaluable patients		
		STAT3 (↑)	STAT3 (↓)	*P* value	p27 (↑)	p27 (↓)	*P*
Age (year)	54.6, 17-80			0.45			0.781
n < 60	22 (53.65)	12 (54.54)	10 (45.45)		8 (36.36)	14 (63.63)	
n ≥ 60	19 (46.35)	12 (63.15)	7 (36.84)		9 (47.36)	10 (52.63)	
Sex				0.27			0.312
Female	13 (31.7)	8 (61.53)	5 (38.46)		3 (23.07)	10 (76.92)	
Male	28 (68.2)	16 (57.14)	12 (42.85)		14 (50)	14 (50)	
Blast percentage				0.37			0.117
n < 40	12 (29.26)	6 (50)	6 (50)		3 (25)	9 (75)	
n ≥ 40	30 (70.74)	18 (60)	12 (40)		15 (50)	15 (50)	

Abbreviation: AML, acute myeloid leukemia
aValues are expressed as No. (%) or median, range
bStatistically significant values: P < 0.05

Table 4. The evaluation of the gene expression of HOTAIR in AML patients [a,b]

Clinical variables	Total Patients, 41 (100)	Evaluable patients		
		HOTAIR (↑)	HOTAIR (↓)	*P*
Age (year)	54.6, 17-80			0.55
n < 60	22 (53.65)	15 (68.81)	7 (31.81)	
n ≥ 60	19 (46.35)	14 (73.68)	5 (26.31)	
Sex				0.7
Female	13 (31.7)	8 (61.53)	5 (38.46)	
Male	28 (68.2)	21 (75)	7 (25)	
Blast percentage				0.41
n < 40	12 (29.26)	7 (58.33)	5 (41.66)	
n ≥ 40	30 (70.74)	22 (73.33)	8 (26.66)	

Abbreviation: AML, acute myeloid leukemia
aValues are expressed as No. (%) or median, range
bStatistically significant values: P < 0.05

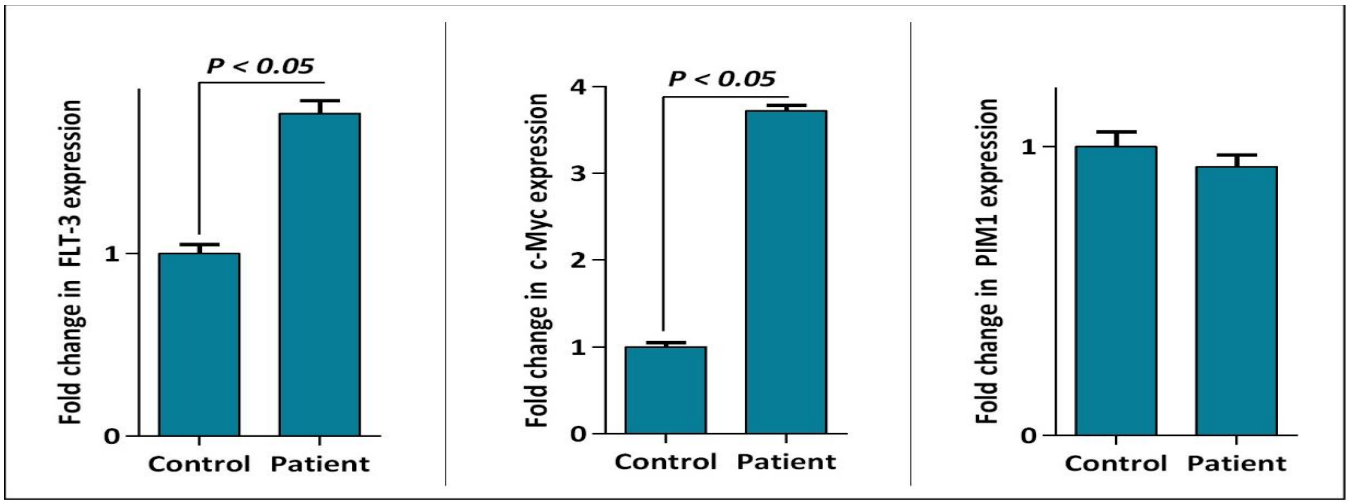

Figure 1. The results of the qRT-PCR analysis revealed that the expression of both FLT-3 and c-Myc increased in AML patients compared to the control group. We also failed to find any significant alteration in the expression level of PIM1 in AML patients. Values are given as mean ± standard deviation of three independent experiments.

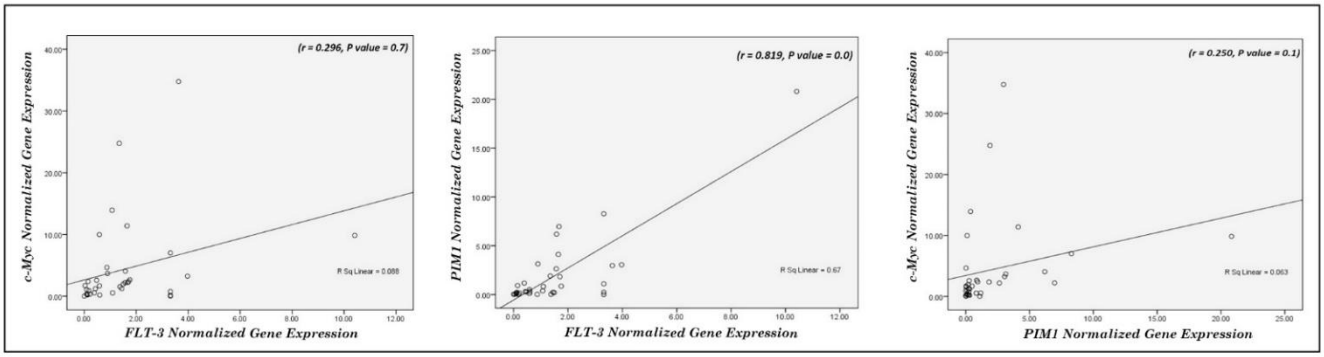

Figure 2. The correlation between the expression level of c-Myc, FLT-3, and PIM1 in AML samples. Normal data distribution was achieved by log 10 computation, and correlation between c-Myc, FLT-3, and PIM1 in AML was determined in 41 AML patients. Values are given as mean ± standard deviation of three independent experiments.

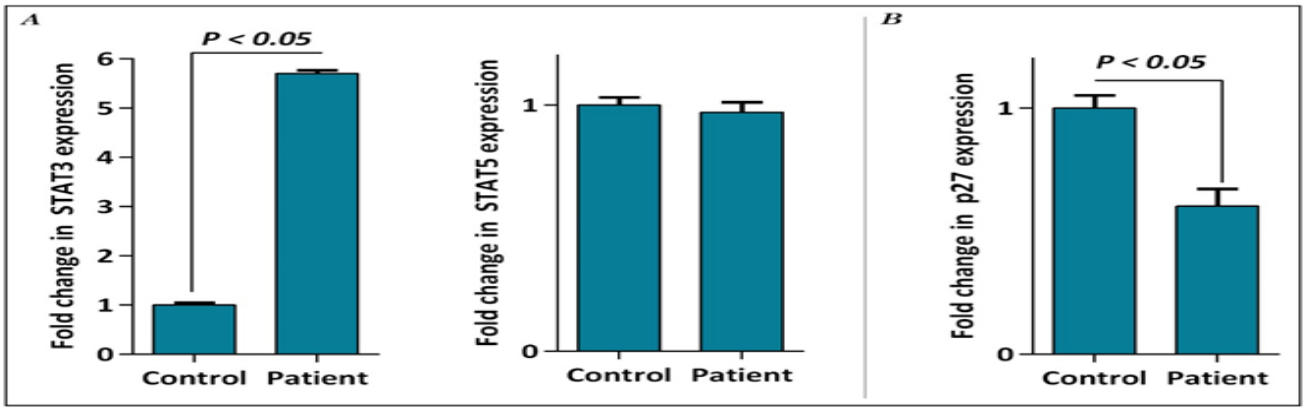

Figure 3. The gene expression analysis results revealed that while the expression of STAT3 significantly increased in AML patients (A), the expression of p27 (B) remarkably diminished in the patients. Values are given as mean ± standard deviation of three independent experiments.

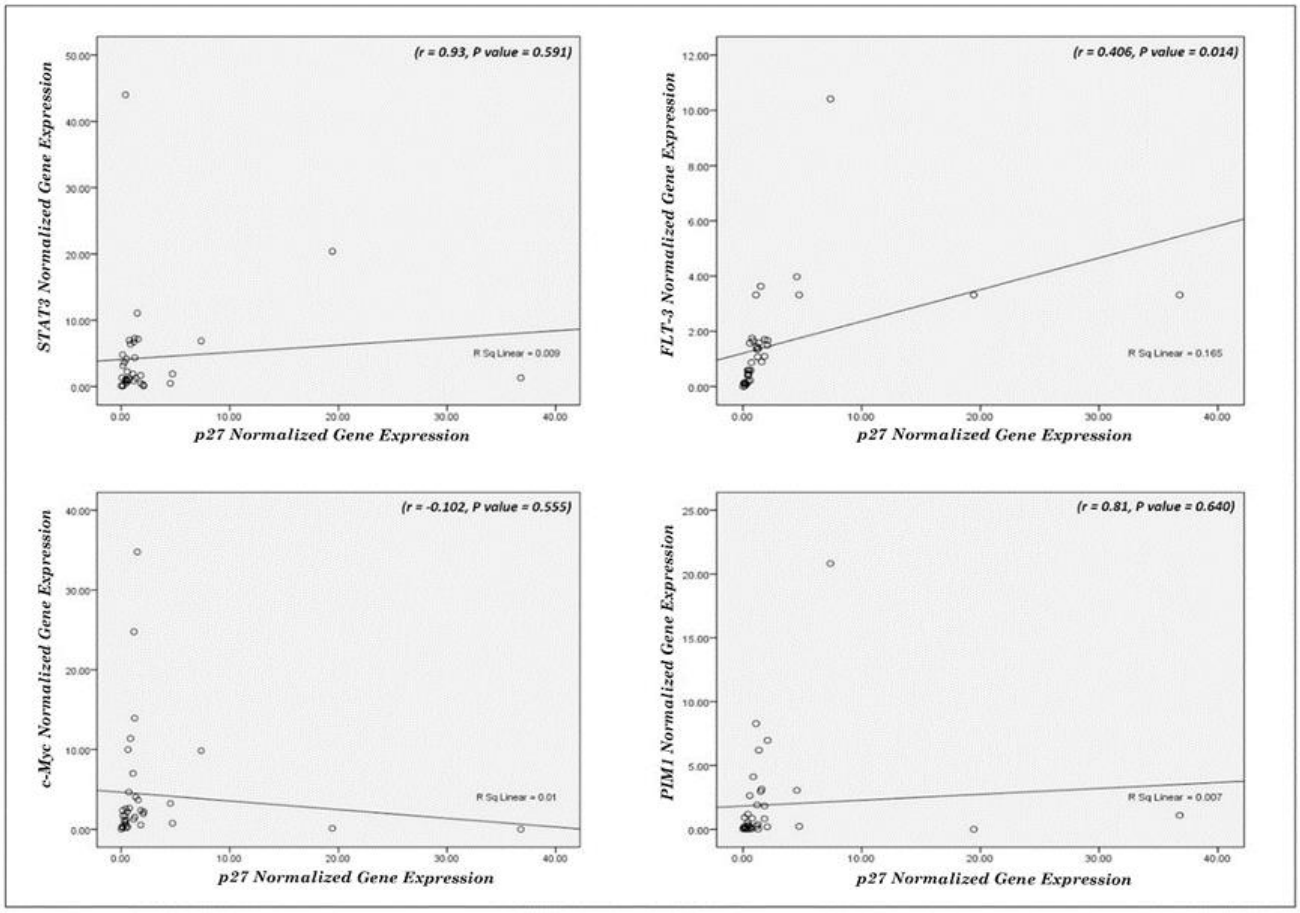

Figure 4. Evaluating the correlation between STAT3, FLT-3, c-Myc, p27 and PIM1 revealed that there is a correlation between p27 and STAT3 expression in AML patients. Values are given as mean ± standard deviation of three independent experiments.

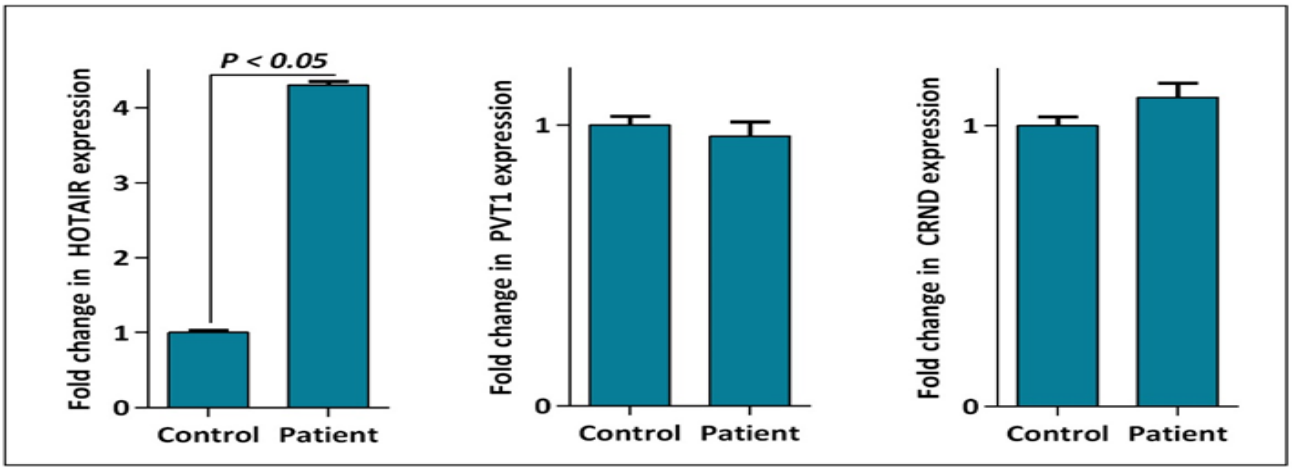

Figure 5. Among different lncRNA, the results of the qRT-PCR analysis showed that the expression of HOTAIR increased more significantly in AML patients as compared to the control group. Values are given as mean ± standard deviation of three independent experiments.

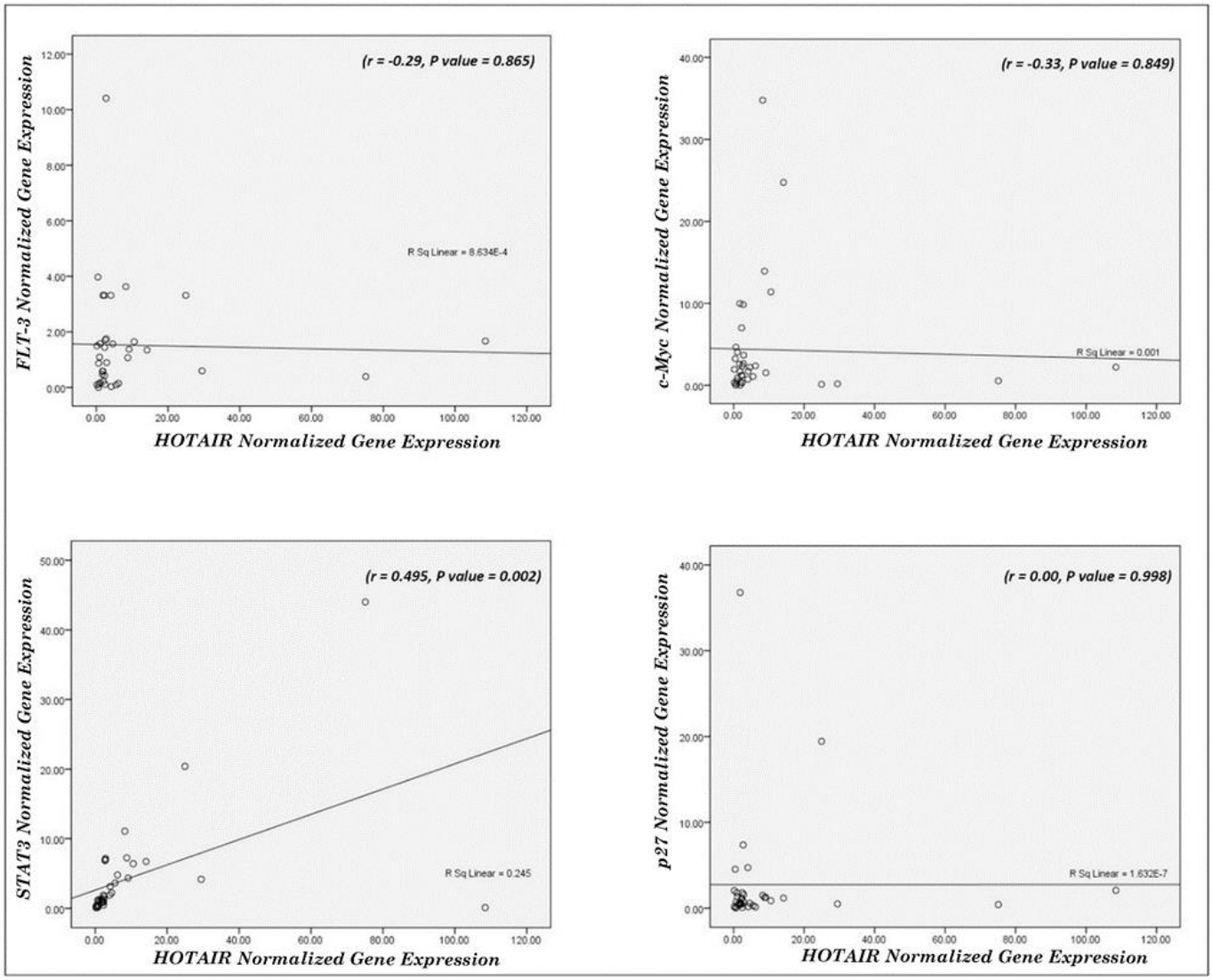

Figure 6. Evaluating the correlation between HOTAIR, c-Myc, STAT3, p27, and FLT3. The results of the Pearson test indicated that HOTAIR had a significant correlation with STAT3 (r = 0.495, $P \leq 0.002$). Values are given as mean ± standard deviation of three independent experiments.

DISCUSSION

AML is one of the common malignancies worldwide that annually threatens the lives of many patients. [15] Despite attempts to treat the disease, the number of patients losing their lives to the disease relapse or the incidence of secondary malignancies is still rising. The heterogeneous nature of AML is one of the main reasons that restrict the ability of the anti-cancer drugs[16]. Despite the attempts to improve the treatment strategies of the disease that might increase the overall survival of the patients, finding new diagnostic markers could be more helpful to AML patients. Besides, finding new molecules could also give another dimension to the basic knowledge of AML. This study aimed to investigate the expression profile of several genes and long non-coding RNAs in newly diagnosed AML patients to not only determine the association between gene expression but also to evaluate through which mechanisms these genes could participate in the development of AML.

Among different molecules participating in the pathogenesis of AML, FLT-3 sustains the survival of leukemic cells by interacting with the BM microenvironment[17]. Although the involvement of FLT-3 in the development of AML is well-established, the evidence supporting this receptor's participation in the early stages of leukemogenesis is minimal. Grag et al. indicated that the incidence of somatic mutations in FLT-3 is not enough for AML development, and other genetic factors are required to convert a myeloid progenitor into leukemic cells[18]. In another study, FLT-3 mutations were reported in relapsed AML patients and not in newly diagnosed patients[19]. In contrast, we found that among 41 newly diagnosed AML patients, 19 patients (46.34%) displayed over-expressed FLT-3. This finding was in agreement with the results of the previous study indicated that 30% of AML patients have over-expressed FLT-3[20]. Moreover, it was indicated that the expression of c-Myc, one of the well-known partners of FLT-3, was elevated in AML patients. Also, no correlation was found between the expression of either FLT-3 or c-Myc and some patient parameters, such as sex, age, and the percentage of the blasts, suggesting that probably the alteration in the expression of these genes is a fundamental event in the leukemogenesis. Having established that both FLT-3 and c-Myc might have important roles in the development of AML, it was of particular interest to investigate the mechanisms through which FLT-3 might regulate the expression of this transcription factor. One of the best suspicion was STAT pathway, as previous studies suggested that upon activation of FLT-3 on leukemic cells, this receptor activates STAT3 through STAT5 phosphorylation and thereby increase the expression of proliferative target genes[21]. Spiekermann et al. also reported that FLT-3 activates STAT5 through the recruitment of the MAPK pathway[22]. Herein, in this study, while the expression of STAT3 was over-expressed, the expression of p27, a transcription factor involved in cell proliferation, was decreased in AML patients. Moreover, the results of the Pearson correlation suggested that there is a meaningful correlation between FLT-3 and p27. In agreement with the present study's finding, it has also been indicated that the over-expression of FLT-3 in acute lymphoblastic leukemia (ALL) cells repress the expression of p27 by increasing the expression of TGF-β[23].

LncRNAs are a group of non-coding RNAs whose regulatory roles in the pathogenesis of human cancers have positioned them as valuable cancer markers[24-26]. Our results showed the overexpression of HOTAIR in AML patients compared to the control group. HOTAIR is a critical lncRNA, and its expression has a tight association with the development of a wide variety of cancers, including breast cancer, lung cancer, colorectal cancer, and hematologic malignancies[27]. In colorectal cancer, it has been reported that HOTAIR overexpression increases the proliferation of cancer cells and induces drug resistance through miR-203a-3p-mediated regulation of B-catenin and GRG5[28]. In lung cancer, the elevation in HOTAIR expression was suggested to be associated with a higher risk of metastasis[29]. It has also been indicated that HOTAIR increases the expression of proliferative-related genes in ovarian cancer cells[30]. The results showed a positive correlation between the expression of HOTAIR and STAT3, suggesting that this lncRNA probably promotes the proliferation of leukemic cells through

interacting with STAT3. In similarity, it has been suggested that HOTAIR increases the cell proliferation of HeLa, CaSKi, ME-180, and HT-3 through recruiting STAT3[31].

CONCLUSION

This study suggested that while the expression of FLT3, c-Myc, STAT3, and HOTAIR increased, the expression of p27 decreased significantly in AML patients compared to the control group. Moreover, since the attempt to find any association between the expression of the genes mentioned above and the patient's age, sex, and the percentage of blasts failed, it can be concluded that these genes probably play a critical role in the early stages of leukemogenesis. Furthermore, a correlation was found between the expression of FLT-3 and p27 and between the expression of HOTAIR and STAT3. Accordingly, one of the straightforward interpretations of our results is that probably FLT-3 overexpression induced c-Myc-mediated suppression of p27. HOTAIR also increased the proliferative capacity of leukemic cells through up-regulating STAT3. Taken together, the results of gene profile analysis proposed that studying the expression of HOTAIR, FLT-3, c-Myc, STAT3 and p27 would be helpful in AML patients and each of these genes could be pharmaceutically targeted in the treatment strategies.

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Ethics statement

All patients and volunteers signed written informed consent forms, and the protocol was reviewed and approved by the Institutional Review Board at Shahid Beheshti University of Medical Sciences. All methods were carried out following national regulations and the Declaration of Helsinki.

CONFLICTS OF INTEREST

The authors declare that they have no conflict of interest.

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