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NF-α, IL6, IL1-α, IL-10 and TGF-β1 Gene Expression in Iranian Acute Myeloid Leukemia Patients

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ABSTRACT

Acute myeloid leukemia is a heterogeneous clonal disorder of blood-producing cells that are among the most common malignant disorders in adults. The purpose of this study was to examine the gene expression of TNF-α, IL6, IL1-α, IL-10, and TGF-β1 at diagnosis in acute myeloid leukemia. The current study included 50 patients with acute myeloid leukemia and 50 control subjects. ELISA was used to determine the serum concentrations of TNF-α, IL6, IL1-α, IL-10, and TGF-β1. After cDNA synthesis and RNA extraction, the expression of TNF-α, IL6, IL1-α, IL-10, and TGF-β1 genes was determined using real-time PCR (ΔΔCT computational). Statistical analysis was conducted with Statistical Package for the Social Sciences 19, and $P<0.05$ was deemed statistically significant. The mean serum levels of TNF-α, IL6, IL1-α, IL-10, and TGF-β1 were lower in patients with acute myeloid leukemia than in the control group. In addition, cytokine mRNA expression in peripheral blood mononuclear cells was significantly lower in AML patients compared to the control group ($p<0.0001$). Changes in TNF-α, IL6, IL1-α, IL-10, and TGF-β1 were associated with acute myeloid leukemia, according to the findings. The current study supports the use of cytokines as diagnostic biomarkers for acute myeloid leukemia.

Keywords: Autoimmune disorders, Hematologic manifestations of systemic diseases, Lymphocytes, Pediatric Leukemia

INTRODUCTION:

Acute myeloid leukemia (AML) phenotypically and genotypically is a heterogeneous disease associated with the accumulation, proliferation and resistance to apoptosis of myeloid hematopoietic precursor cells as well as the inhibition of their differentiation in the bone marrow and peripheral blood. In this disease, leukemic cells disrupt the maturation of normal myeloid, erythroid and megakaryocytic precursor cells [1, 2]. Leukemia is the fifth most common cancer in the world and the most common cancer after gastric cancer in Iran [3, 4]. AML is the most common form of acute leukemia in adults, accounting for 0.6% of all cancers. This type of cancer is one of the malignancies that can lead to death quickly if left untreated. Approximately 18,300 people are annually diagnosed with AML in Europe [5, 6]. The AML incidence is about 3.5 per 100,000 individuals per year and is more prevalent in men than women [7]. The incidence of AML increases with age and the mean age at diagnosis has been reported 67 years. Since the majority of the Iranian population is in the age range of 20-45 years; therefore, the highest incidence of this type of cancer is in this age range. As this group is more exposed to other risk factors such as environmental factors as well as noise and chemical pollution, they are at increased risk of developing the disease [3, 4]. Due to the genetic changes in the blood precursor cells, the natural growth and differentiation of these cells are altered, leading to the accumulation of a large number of abnormal and immature myeloid cells in the peripheral blood and bone marrow. These cells are capable of dividing and multiplying but incapable of becoming mature hematopoietic cells [8]. Cytokines are peptide mediators that are involved in regulating immune responses, local-systemic inflammatory responses and healing responses to causative agents. The cytokines play their role via stimulating or inhibiting cell proliferation and differentiation [9]. Several studies have demonstrated that the cytokines produced by leukemic cells control the growth of AML cells in an autocrine or paracrine manner. In contrast to normal hematopoietic cells, many AML patients' leukemic blasts express cytokines such as SCF, IL-1, G-CSF, GM-CSF, IL-8, IL-6 and TNF-α constitutively. [8-11]. The absence of a balance between inflammatory and anti-inflammatory cytokines hinders the immune system's ability to function properly. Recent research indicates that any difference in cytokine levels (high or low) is associated with distinct cancer types [10]. This study aimed to examine the expression of TNF-α, IL6, IL1-α, IL-10, and TGF-β1 genes in acute promyelocytic leukemia at the time of diagnosis and after treatment.

Methods:

In this case-control study, peripheral blood samples were collected from 50 AML patients at the time of diagnosis in 2015-2018. Blood samples were also taken from 50 healthy controls. Before any sampling, the consent form was obtained from all participants.

2.1. Measurement of serum cytokines

The ELISA kit (Quantikine® ELISA Kit) was used to measure serum cytokines. The principle of this kit is based on direct ELISA and its sensitivity is 2 ng/ml. In this method, the unlabeled antigens present in the serum of patients, controls and standards compete with a biotin-labeled antigen for binding to restricted antibodies cultured at the bottom of the wells in a 2.5-hour incubation. Then, the enzyme-substrate, Tetramethylbenzidine (TMB), was added to the medium after 4-stage washing, removing free antibodies from the medium. The conversion reaction from the substrate to the solution, initiated after a 15-minute incubation was terminated by adding the stop solution to the reaction, and within 20 minutes the optical absorption was read using a spectrophotometer at a wavelength of 450 nm against control at 630 nm. The standard diagrams were drawn according to the standard optical absorption, and the optical absorption of the test and control groups was compared with those diagrams. The higher the optical absorption of the solution indicated the higher the number of studied cytokines in the serum or control.

2.2. Isolation of mononuclear cells

Each patient and healthy subject provided approxi-

mately 10 ml of EDTA-treated peripheral blood for analysis. On Ficoll, the mononuclear cells were isolated using a density gradient.

2.3. Extraction of total RNA from mononuclear cells and cDNA construction:

Total RNA was extracted using an extraction solution (RNX-plus CinnaGen) based on the manufacturer's instructions. In the present study, the Fermentase RevertAid ™ First Strand cDNA Synthesis Kit was applied to construct the cDNA.

Then, 4 μg of total RNA was used as a template for cDNA synthesis. For the reverse transcription reaction, the reaction mixture was incubated for 5 min at 45 ° C, 69 min at 24 ° C and 5 min at 75 ° C.

2.4. Real-time polymerase chain reaction (RTPCR)

The RTPCR and Syber Green PCR Master Mix were used to evaluate the expression levels of TNF-α, IL6, IL1-α, IL-10, and TGF-β1 genes in the studied persons. A dedicated primer pair designed by the research team via the NCBI site and Beacon Designer 8 was used in this method. The dedicated primer pair was also applied to evaluate the expression level of GAPDH gene as a housekeeping gene (Table 1). In a final volume of 25 μL, the quantitative RTPCR (qRTPCR) reaction mixture contained 12.5 μL of 1 × SYBR-green master mix (Applied Biosystems), 100 ng of diluted cDNA (1.5 μL), 2 μM of each appropriate primer (1 μL), and 9 μL of infusion water. Using 45 cycles on a Corbet Rotor-Gene 6000, the RTPCR quantification was performed (Corbett, Australia). Initial denaturation at 95 °C for 2 minutes was followed by 45 cycles of denaturation at 95 °C for 10 s, primer annealing at 60 °C for 15 s, and extension at 72 °C for 20 s. At the end of an amplification reaction, the analysis of melting temperature was also performed from 55 °C to 95 °C at a rate of 0.1 °C/s. The relative expression changes of the target genes (TNF-α, IL6, IL1-α, IL-10 and TGF-β1) relative to that of the control gene (GAPDH) were determined using the cycle of threshold (Ct) values, standard curve analysis, and the 2-ΔΔCt relative quantitative method.

2.5. Statics

Using descriptive statistics and SPSS 16, MedCalc 18.2.1, the values (means ± SD) and statistical calculations were analyzed/performed (MedCalc Software bvba, Mariakerke, Belgium). The Shapiro-Wilk test, the paired t-test, and the Wilcoxon test were used to determine, respectively, the normality of the data, the normal distribution of the variables, and the non-normal distribution of the variables. All p values were two-sided, and $p<0.05$ was deemed to be statistically significant.

Results:

In the current study, 50 patients [31 males (62%)] with AML were studied. Their mean standard deviation was 40.34±16.2, and the median age at diagnosis was 42 years. The mean serum levels of TNF-α, IL6, IL1-α,

Table 1. Primers for qRT-PCR

bp	Primer	Gene
TGF-β1	F: 5'-CGACTACTACGCCAAGGA-3' R: 5'-GAGAGCAACACGGGTTCA-3'	150
TNF-α	F: 5'-CCCAGGCAGTCAGATCATCTTC-3 R: 5'-AGCTGCCCCTCAGCTTGA-3'	85
IL-10	F: 5'-GCCTAACATGCTTCGAGATC-3' R: 5'-TGATGTCTGGGTCTTGGTTC-3'	206
IL6	F: 5'-GGTACATCCTCGACGGCATCT-3' R: 5'-GTGCCTCTTTGCTGCTTTCAC-3'	81
IL1-α	F: 5'- ATTCATCCTGAATGACGCCT-3' R: 5'- ACCCATGTCAAATTTCACTGCTT-3'	215
GAPDH	'F:5'- AAGCTCATTTCCTGGTATGACAACG-3 'R:5'- TCTTCCTCTTGTGCTCTTGCTGG-3	126

Table 2. TNF-α, IL6, IL1-α, IL-10 and TGF-β1 levels in both groups

P value	ctrl	AML	Parameter
N	0.78±0.26	0.52±0.15	TNF-α (Pg/mL)
0.04	54.52±4.9	42.2±5.23	IL6 (ng/mL)
N	12.51±2.8	10.21±2.1	IL1-α (Pg/mL)
0.0049	3.75±0.15	2.23±0.14	IL-10(Pg/mL)
N	2.61±0.74	1.41±0.56	TGF-β1 (ng/mL)

IL-10 and TGF-β1 were lower in AML patients than in the control group (Table 2). In contrast, serum levels of IL6 and IL10 were significantly lower in the experimental group, with a mean of 42.2 ng and 2.23 Pg/mL, respectively (p=0.04 and p=0.0049, respectively). Gene expression of cytokines including TNF-α, IL6, IL1-α, IL-10and TGF-β1 was lower in AML patients than in normal controls, according to the results of an ongoing study (Fig. 1). Table 2. Description of the serum levels of TNF-α, IL6, IL1-α, IL-10, and TGF-β1in patients and controls. Data are shown as mean ± standard deviation. Analysis of qPCR results represented that the expression of the studied interleukins was significantly reduced in AML patients compared to normal controls (Figure 1). The mean fold change of interleukins such as IL6, IL1-α, TNF-α, IL-10 and TGF-β1 was 1.9, 5.76, 4.02, -7.59 and -1.37in AML patients, respectively (Fig. 1A). In addition, the heatmap diagram reported the expression level of the studied cytokines in each individual (Figure 1B).

Discussion:

AML is an invasive hematological disorder characterized by the inhibition of cell differentiation and accumulation of immature myeloid cells in the bone marrow. The present study evaluated the expression level of important cytokines such as TNF-α, IL6, IL1-α, IL-10 and TGF-β1 by ELISA and qPCR in blood mononuclear cells of AML patients. According to the results of the present study, the serum and expression levels of TNF-α, IL6, IL1-α, IL-10 and TGF-β1 declined in AML patients compared to healthy controls. Various studies have displayed differences in the levels of studied cytokines. For example, Reddy et al. in 2007 found that there was a low expression level for IFN-β and TNF-α, whereas there was a high expression level of IL-8 in AML patients compared to healthy controls [12]. However, Gao et al. expressed a high-level expression of TNF-α and IL-1βin AML patients [13]. Moreover, several studies have evaluated the expression profile of cytokines in patients with AML and other diseases [14, 15]. In the current study, the IL 6 level had a significant decrease in AML patients compared to the control group. Decreased levels of this cytokine can be attributed to leukemia in the category of non-inflammatory diseases because numerous studies like the study of Sanchez-Correa B et al. have demonstrated that these cytokines are proinflammatory, enhance inflammatory conditions such as arthritis and usually do not change much in non-inflammatory conditions [16]. Since the hematopoietic role of this cytokine cannot be ignored; hence, the changes of this cytokine in blood malignancies are not imaginary. The leukemia-inhibiting factor is a multifactorial cytokine that exhibits different functions in various cell types; the important function of this cytokine is to activate four pro-opiomelanocortin gene transcriptions in response to immune stimulation [17]. The results of the ongoing study illustrated that TGF-β1 was reduced in the studied patients compared to healthy subjects. The role of TGF-β1 in various cancers has been elucidated [18]. The analysis of the present study represented that the TGF-β mRNA levels decreased in both B-ALL and TALL groups compared to the control group. Decreased cell-surface expression of

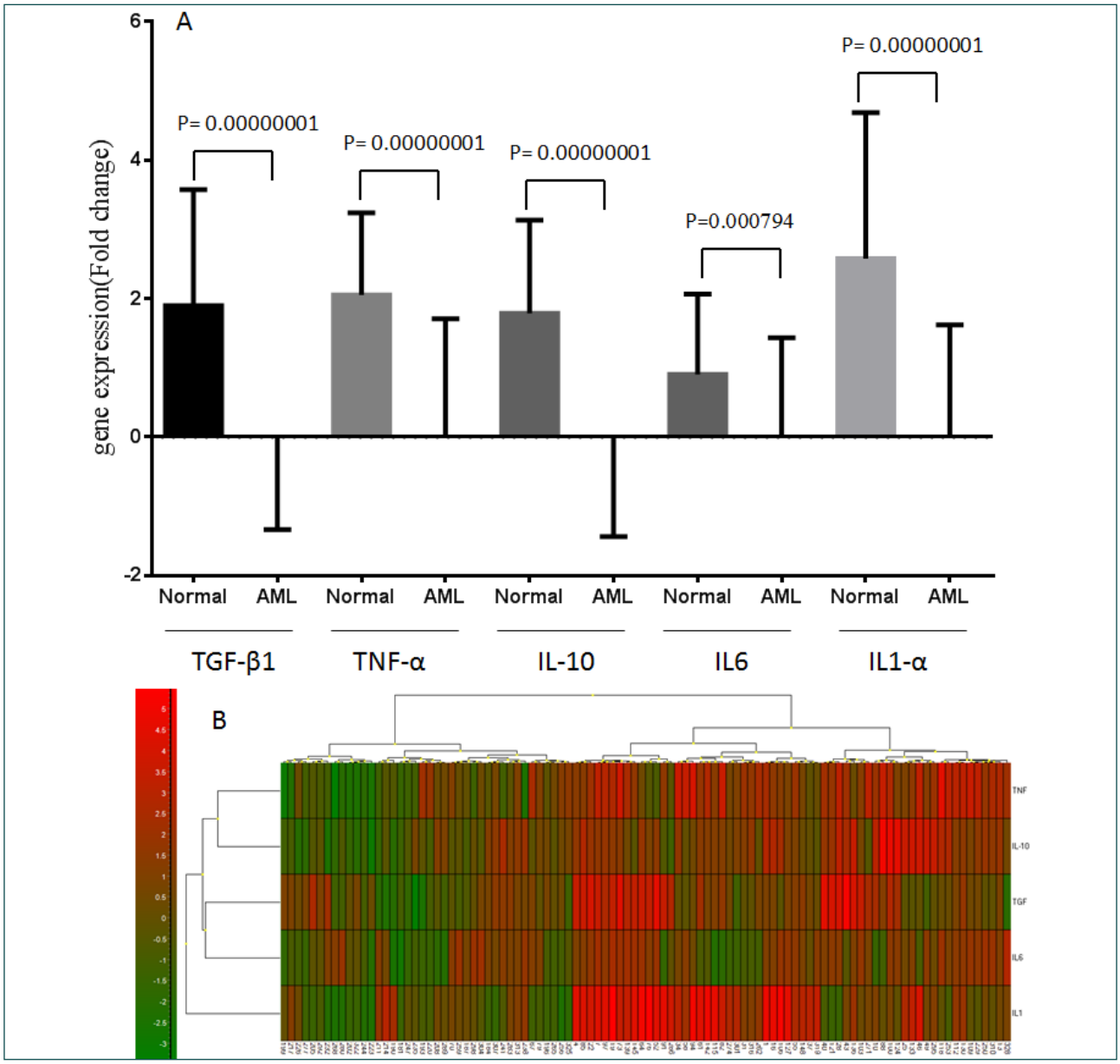

Figure 1A. Comparative qPCR results of TNF-α, IL6, IL1-α, IL-10 and TGF-β1 mRNA expression in AML Cell (A, n=100, with various p values done with t-test)
Figure2B. Comparison of full heatmap between TNF-α, IL6, IL1-α, IL-10 and TGF- genes in AML data indicating the correlation of the mRNA expressions .

TGF-β receptors, mainly TβRI, can be caused by the lack of response of CLL cells to TGF-β [19], but this matter was not studied in the current research. Furthermore, the serum and expression levels of the IL10 gene declined in the studied patients compared to the control group. The IL-4 has antitumor effects and suppresses the growth of acute lymphoblastic leukemia cells; the in vitro proliferation of leukemic cells, particularly the AML subset, is increased by IL-4-producing T lymphocytes [20, 21]. Nevertheless, the in vitro effects of IL-4 and IL-10 do not indicate those in vivo effects [21]. The expression of distinct cytokines like IFN-γ and IL-10 is related to the differentiation and lineage commitment of the leukemic cells [22]. Besides, the serum and ex-

pression levels of the IL1 gene were also lower in the studied patients than in controls. Different studies have suggested the role of IL1 in AML. The leukemic cell proliferation is stimulated through IL-1, β-chemokine and IL-2 of CC subgroup CCL3 (CC-ligand-3) [23]. A cytokine-induced proliferation study has been carried out to evaluate the effect of some single cytokines on the proliferation of patient-derived AML cells [24].

Conclusion:

The results indicated that changes in TNF-α, IL6, IL1-α, IL-10, and TGF-β1 had a significant relationship with AML. Cytokines can also be used as biomarkers in the diagnosis of AML. Further studies with larger sample sizes and mutations in these genes are recommended. Additionally, evaluating the signaling related to these cytokines is important.

Data Availability

On reasonable request, the corresponding author will provide access to the datasets generated and/or analyzed during the current study.

Ethical Approval

The Ethics Committee of Tehran University of Medical Sciences, Tehran, Iran, has approved this study.

Conflicts of Interest

The authors report having no conflicts of interest.

Contributor's Statement: This research was designed and supervised by one of the authors and the data was analyzed and interpreted by another.

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Ethics: Prior to any sampling, the consent form was obtained from all participants.

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