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Change in Programmed Death-1 and Inducible Costimulator Expression in Patients with Acute Myeloid Leukemia Following Chemotherapy and Its Cytogenetic Abnormalities

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Abstract

Background: Programmed death-1 (*PD-1*) and inducible costimulator (*ICOS*) are immune checkpoint receptors participating in tumor immune evasion, which counters the activation signal provided through the T-cell receptor ligation. This study aimed to investigate the relationship between the expression of *PD-1* and *ICOS* on mononuclear cells (MNCs) isolated from the peripheral blood of acute myeloid leukemia (AML) patients and their response to induction chemotherapy. **Materials and Methods:** Peripheral blood samples (5 cc) were collected from 56 AML patients at first diagnosis before and after the induction therapy regimen for AML. PD-1 and *ICOS* expression were analyzed in all patients before and after the standard induction therapy regimen. **Results:** The expression of *PD-1* and *ICOS* significantly decreased (66.7 and 16.3 fold, respectively) in AML patients following chemotherapy compared to its baseline value (P=0.01 and P=0.001, respectively). The expressions of *PD-1* and *ICOS* were significantly different between favorable and poor risk groups. **Conclusions:** Lower *PD-1* and *ICOS* expressions on the surface of MNCs before induction therapy were associated with a better response to treatments. In addition, *PD-1* and *ICOS* expression on MNCs decreased after induction therapy.

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Keywords: Acute Myeloid Leukemia; Programmed Death; Inducible Costimulator; Induction Therapy; Mononuclear Cells

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Introduction

Acute myeloid leukemia (AML) is the most common form of acute leukemia in adults [1]. The principal feature of AML is its variable responses to therapy [2]. Also, new studies have revealed that chemical drug resistance rather than treatment-related mortality is the major cause of treatment failure in AML [3]. The general therapeutic strategy in patients with AML is intensive induction chemotherapy followed by appropriate post-remission therapy after achieving complete remission [4]. The mainstay of initial induction therapy for AML is cytarabine in combination with an anthracycline (i.e., daunorubicin or idarubicin) [5]. Although short-term complete remission is achieved after standard chemotherapy in most cases, long-term remission is obtained in less than 50% of the patients [6]. Hence, some efforts have been made to target the immune system as a novel therapeutic modality for treating AML because of the proven role of the immune system in the control of AML [7]. These efforts include treatment options such as multispecific antibody (Ab) constructs and treatments targeting immune checkpoint inhibitors [7, 8]. *PD-1*, an immune checkpoint receptor inducibly expressed on B-cells, T-cells, and activated monocytes, belongs to the immunoglobulin (Ig) superfamily [9]. Recently, the crucial role of *PD-1* signaling has been identified in regulating peripheral tolerance and autoimmunity by inducing a coinhibitory signal in activated T-cells leading to T-cell apoptosis and functional exhaustion [10]. Recent evidence indicates that PD ligand 1 (PD-L1) expression may be related to tumor growth promotion and apoptosis induction [11]. Also, it has been shown that blocking the PD-L1/*PD-1* pathway using anti-PD-L1 or anti-*PD-1* monoclonal Ab results in the inhibition of tumor growth and boosting of antitumor immunity [11]. Also, it has been proposed that the *PD-1*/PD-L1 mechanism plays a role in the interaction of AML blasts with the immune system in mouse models [12]. Increased expression of PD-L1 was identified in blast cells from AML patients exposed to immune response, pathogens, and sometimes upon relapse, and thus it was considered as an immune escape molecule [13]. However, in another study, it was concluded that *PD-1* was not related to the survival of AML patients [14]. According to several studies, *ICOS* appears to play a role in solid organ graft rejection. *ICOS* inhibition improved cardiac transplant survival, according to Harada *et al*. while liver transplant survival was improved in another trial [15]. Fewer studies have looked at the involvement of the *ICOS* pathway in bone marrow (BM) transplantation, while one study utilized non-irradiated parent-into-F1 models. Burlion *et al*. investigated that *ICOS* inhibition decreased T helper 2 (Th2)–mediated chronic graft-versus-host disease (GVHD) but increased Th1–mediated acute GVHD [16]. In additional trials, *ICOS* blocking has also been beneficial for GVHD prevention, therapy, and allogeneic BM graft promotion [16]. Hence, *ICOS* expression best characterizes inflammatory effector T-cells 26, imply that the *ICOS* pathway could be a promising therapeutic target [15]. Profiling the surface expression of *PD-1* on mononuclear cells (MNCs) isolated from peripheral blood (PB) of AML patients may represent valuable data anticipating the patient's response to the standard induction chemotherapy regimen. Hence, this study aimed to assess the relationship between the expression of *PD-1* on MNCs isolated from the PB of AML patients and their response to induction chemotherapy.

Materials and Methods

Patients and Design

Fifty-six newly diagnosed adult AML patients who were referred to Namazi Hospital (Shiraz, Iran) were selected. PB samples (5 cc) were collected from the patients before starting treatment. WHO criteria were used to diagnose and evaluate the morphology and cytochemistry of BM samples collected from the patients. Complete blood count, blast percentage, and hemoglobin (Hb) levels were also collected. All patients received a standard induction therapy regimen for AML consisting of daunorubicin (45 mg/m^2) on days one to three, and cytarabine

(100-200 mg/m^2) on days one to seven, followed by high doses of a cytarabine-based consolidation phase (cytarabine 3 g/m^2 every 12 hours for three days, repeated for two to three cycles) except for promyelocytic AML patients for whom ATRA was added to this regimen. A second BM examination was performed after induction therapy on days 17-21. Post-induction chemotherapy samples from PB were also collected from all patients. The isolation of MNCs from whole PB samples was performed by Ficoll density gradient centrifugation. Complete remission was considered as no evidence of extramedullary leukemia, no Auer Rods, neutrophil and platelet recoveries to 1-l0^9 and 100•10^9/l, respectively, and <5% blasts in a BM aspirate (irrespective of cellularity) [17, 18].

Determined Cytogenetic Status

The National Comprehensive Cancer Network (NCCN) guidelines were used to stratify cytogenetic status [19]. The presence of t (8;21), t (15;17), or inv/t (16), (nucleolar phosphoprotein B2) NPM1 or mutated (CCAAT/enhancer binding protein-alpha) CEBPA without FLT3-ITD was considered as a favorable risk abnormality. NCCN guidelines categorize an intermediate risk abnormality with the presence of normal cytogenetics, trisomy 8, t (9;11), t (8;21), inv (16), and t (16;16) with the cKIT mutation. A poor risk abnormality was defined by the presence of t11q23 (other than t [9;11]), del5/5q, del7/7q aberrations, t (6;9), inv3, t (3;3) aberrations, or a complex karyotype (three or more numerical and/or structural abnormalities), and normal cytogenetics with FLT3-ITD mutation.

RNA Isolation and cDNA Synthesis

The total RNA was extracted by RNX-Plus solution (CinnaGen, Tehran, Iran). The quantity of the extracted RNA was evaluated by the NanoDrop 2000c (Thermo Scientific, Waltham, MA, USA) that measured the optical density 260/280, and the quality of the extracted RNA was assessed by running 3 µL on 1% agarose gel. The quality of RNAs was indicated by the lack of a smear on the lower part of the gel (a smear indicates RNA degradation) and by the presence of 28S ribosomal RNA twice as intense as 18S rRNA. After obtaining a good-quality total RNA, cDNA was synthesized using Prime Script RT Reagent Kit (Takara, Japan) according to the manufacturer's guidelines.

Real-time Polymerase Chain Reaction (PCR)

The real-time PCR using SYBR green (Applied Biosystems, USA) was performed to quantitatively analyze *PD-1* and *ICOS* mRNA expression profiles. The glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene was used as an internal control for minor fluctuations. The PCR program and primer sequences are summarized in Table 1. The melt curve was analyzed to confirm the specificity of the reaction at the end of the program. The results for the target genes were measured as fluorescent signal intensity and normalized to the internal standard gene GAPDH.

Cycle threshold (Ct) values, which are inversely proportional to the original relative expression level of the gene of interest, were

Table 1. The Primers and PCR Conditions

Genes	Primer sequences (5'-¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬¬>3')	Thermocycling condition
PD-1	F: CTTAGACTCCCCAGACAGG	95 °C for 2 min, 40 cycles of 95 °C for 30 sec, 60 °C for 20 sec, and 70 °C for 30 sec
	R: GCGTTGTCCCCTTCGGT	
ICOS	F: TTGAACACTGAACGCGAGGA	
	R: GCAGAACCATTGATTTCTCCTGT	
GAPDH	F: GGACTCATGACCACAGTCCA	95 °C for 2 min, 40 cycles of 95 °C for 30 sec, 57.5 °C for 20 sec, and 70 °C for 30 sec
	R: CCAGTAGAGGCAGGGATGAT	

PD-1: Programmed death-1; **ICOS**: Inducible costimulator; **GAPDH**: Glyceraldehyde 3-phosphate dehydrogenase

used to calculate the relative quantitation. The changes in the relative expression levels of *PD-1* and *ICOS* mRNA were calculated by $2^{-\Delta\Delta Ct}$ method, where ΔΔCt=(ΔCt [before chemotherapy]-ΔCt [after chemotherapy]) and ΔCt=(Ct [sample]-Ct [housekeeping gene]).

Ethical Considerations

All the patients signed the written informed consent by the Helsinki protocol of 1975, and the study was approved by the Ethical Committee of Shiraz University of Medical Sciences (code: 1394-01-32-10603).

Statistical Analysis

The data were analyzed by SPSS Statistics for Windows, version 15 (SPSS Inc., Chicago, Ill., USA). The differences in the mean expression level of *PD-1* and *ICOS* before and after chemotherapy were compared via paired t-test. Also, the mean expression level of *PD-1* and *ICOS* regarding laboratory data were analyzed by the chi-square test. The expression level of *PD-1* and *ICOS* were compared among the patients according to their response to chemotherapy treatment, cytogenetic aberration, and French-American-British (FAB) subtypes by an independent t-test. A P-values less than 0.05 were considered as significant differences.

Results

The relative mRNA expression level of *PD-1* and *ICOS* from 56 patients (30 males and 26 females) was evaluated by real-time PCR. Patient characteristics are listed in Table 2. The median age of patients was 49 years (ranged 20-86 years). The median count of white blood cell (WBC), Hb, and platelet were 27.3×10^9 (ranged 1.3-147), 8.1 g/dl (ranged 3.8-14.7), and 54×10^9 (ranged 3-267). The mean baseline PB and BM blast percentages were 65% (ranged 18-90) and 68% (ranged 20-98), respectively.

Change in PD-1 and ICOS Expression in AML Patients Following Chemotherapy

Based on the results, the expression of *PD-1* and *ICOS* significantly decreased (66.7, 16.3 fold) in AML patients following chemotherapy compared to its baseline value (Figure-1 and -2).

Also, *PD-1* and *ICOS* expression levels were not significantly higher in AML patients who did not respond to chemotherapy than in those who responded to chemotherapy (Figure-1 and -2).

PD-1 and ICOS Expression According to Cytogenetic Abnormalities

Delta Ct values for *PD-1* and *ICOS* mRNA expression levels were compared among favorable (11 cases), intermediate (23 cases), and poor (22 cases) risk groups. The expression of *PD-1* and *ICOS* were significantly different between favorable and poor risk groups (favorable vs. poor: 0.356 vs. -0.293, P=0.03, and -1.2 vs. -0.2.3, P=0.002, respectively). However, the expression of *PD-1* and *ICOS* were not different between favorable and intermediate groups (favorable vs. intermediate: 0.365 vs. 0.492, P=0.568, and 1.2 vs. 1.1, P=0.59). The expressions of *PD-1* and *ICOS* were not different between intermediate and poor risk groups, although the

Table 2. Patient Characteristics

Variables	No. of patients (%)
Sex	
Male	30 (53.5)
Female	26 (46.5)
AML types	
With minimally differentiated	3 (5.3)
Without maturation	5 (8.9)
With maturation	26 (46.4)
Myelomonocytic leukemia	13 (22.7)
Monoblastic	9 (16)
Response rate	
CR	30 (53.5)
NCR	26 (46.5)
Cytogenetic/molecular risk	
Favorable	22 (39.3)
Intermediate	23 (41.1)
Poor	11 (19.6)

AML: Acute myeloid leukemia; **CR**: Complete response; **NCR**: Non-complete response

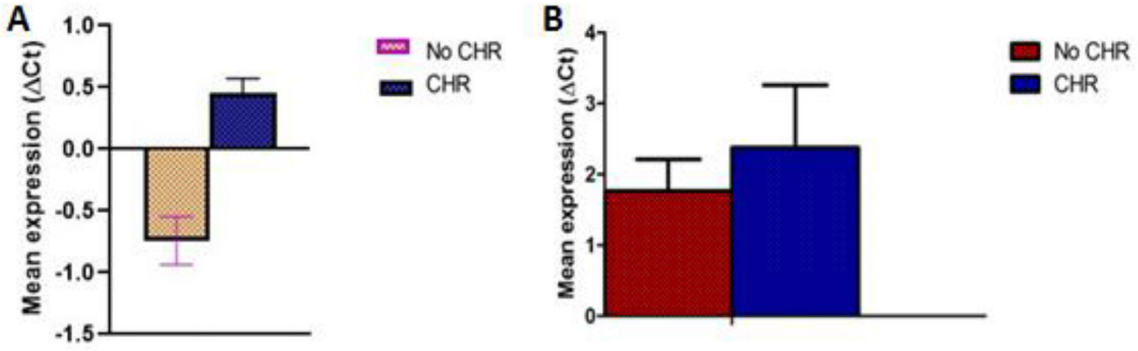

Figure 1. Changes in programmed death-1 (*PD-1*) (**A**) and inducible costimulator (*ICOS*) (**B**) expressions following chemotherapy

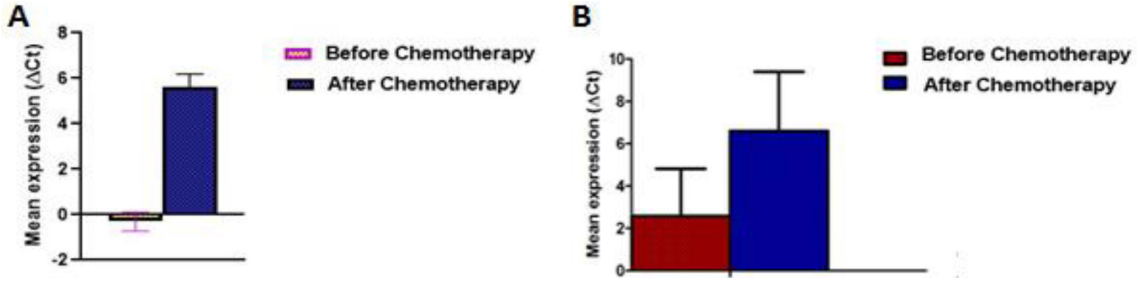

Figure 2. Programmed death-1 (*PD-1*) (**A**) and inducible costimulator (*ICOS*) (**B**) expressions in AML patients and their response to treatment

poor risk group exhibited a higher expression of *PD-1* and *ICOS* (0.492 vs. -0.293, P=0.91, and 0.1 vs. -0.2.3, P=0.72).

PD-1 and ICOS Expression in AML Patients and Their Response to Treatment

In the favorable risk group, the expression of *PD-1* and *ICOS* were different in seven responders cases (Ct=-0.761 and Ct=-0.861, respectively) vs. four non-responders (Ct=2.3 Ct=1.5, respectively) to induction therapy (P=0.023 and P=0.04, respectively). No differences in *PD-1* and *ICOS* expression were found in the complete response (CR) group (eight cases, Ct=-0.796 and Ct=-0.921, respectively) vs. the non-CR (NCR) group (14 cases, Ct=-0.147 Ct=-0.864, respectively) to the first anthracycline-based induction therapy in the poor risk group (P=0.82 and P=0.92, respectively). In the intermediate risk group, *PD-1* and *ICOS* did not show significant differences between the CR (14 cases) and the NCR groups (nine cases) (CR vs. NCR; -0.845 vs. -0.744, P=0.969, and 0.652 vs. -0.951, P=0.72, respectively).

Expressions of *PD-1* and *ICOS* were not significantly different among the types of AML (P=0.861). Also, there was no significant association between *PD-1* and *ICOS* expression and cytogenetics. No significant differences were observed between mean *PD-1* and *ICOS* expression and sex, Hb, WBC count, and blood group (P>0.05).

Discussion

In this study, we aimed to identify the clinical role of *PD-1* gene expression level in response to induction chemotherapy to find a possible prognostic marker for patient risk stratification at first diagnosis leading to the selection of a suitable treatment plan. Lower *PD-1* expression before induction therapy was associated with a better response to induction therapy. In addition, *PD-1* expression decreased after induction therapy.

Recently, the important role of the *PD-1*/PD-L1 signaling pathway in tumor immune evasion has gained widespread attention [20]. *PD-1* is an immune checkpoint acting as a negative regulator of activated

T-cells [20]. However, the up-regulation of surface expression and inhibitory functions of these receptors in T-cells of the tumor microenvironment leads to T-cell dysfunction and, consequently, impaired antitumor immunity [17, 21]. Animal models of tumor growth have demonstrated that this immune evasion mechanism operating in cancer can be suppressed by blocking checkpoint receptors with Abs leading to antitumor immunity restoration and prevention of tumor progression [22, 23]. Hence, it seems that some human tumors, including hematological malignancies, may also benefit from checkpoint inhibition with anti-*PD-1* Ab. Pembrolizumab and nivolumab are anti-checkpoint Abs targeting *PD-1*. They have been approved for melanoma, and their efficacy for the treatment of other cancers, including hematological malignancies is currently being intensively investigated [24].

On the other hand, increased Treg frequency, which is often observed in AML patients at various stages of diagnosis and treatment, has been correlated with poor prognosis and leukemia relapse [25, 26]. Also, it has been shown that the development of peripheral (inducible) Treg (pTreg) from human Th1 cells is conducted by the expression of PD-L1 in the tumor microenvironment. Therefore, the blockade of the *PD-1*/PD-L1 pathway in the tumor microenvironment can be correlated with the arrest of Treg-mediated immunosuppression [17, 27]. Some previous studies have demonstrated the effective role of anti-*PD-1* Ab therapy in refractory and relapsed Hodgkin's lymphoma, melanoma, and advanced multiple myeloma [24, 28, 29]. Also, the impairment of the T-cell function caused by high *PD-1* expression on $CD4^+$ T-cells in diffuse large B-cell lymphoma has been associated with a poor prognosis. Furthermore, compared to *PD-1* negative patients, reduced event-free survival and overall survival were found in patients with positive *PD-1* expression within the tumor microenvironment [30]. More importantly, increased *PD-1* expression in PB MNCs subsets in patients with renal cell carcinoma and the observed correlation between those expression levels in effector T-cells and the disease stage may indicate the probable role of *PD-1* expression as a biomarker for predicting disease severity and prognosis [17, 31].

The current study explored the relationship between *PD-1* expression on the surface of MNC isolated from the PB of AML patients and their response to induction chemotherapy. Lower *PD-1* expression before induction therapy was found to be associated with a better response to it. In addition, *PD-1* expression in the MNCs decreased after induction therapy. Because one of the most important prognostic factors in AML patients is their response to the first induction therapy [32] and based on the obtained results of this study, it can be suggested that higher *PD-1* expression in AML patients is associated with poor prognosis. Our data are in line with some previous studies concerning the relationship between the high level of *PD-1* expression and poor prognosis. Muenst *et al*. found that *PD-1*-positive lymphocytes >23/mm^2 in classical Hodgkin lymphoma patients were associated with poor prognosis compared to *PD-1*-positive cells <23/mm^2 [33]. Also, *PD-1* expression on the surface of $CD4^+$ or $CD8^+$ T-cells within follicular lymphoma was suggested as an unfavorable prediction factor for transformation, thereby leading to poor prognosis [34, 35]. Furthermore, a higher number of relapses was associated with high *PD-1* expression in AML patients [19]. In contrast, Yang *et al*. demonstrated that high *PD-1* expression on the surface of $CD4^+$ or $CD8^+$ T-cells within follicular lymphoma did not affect on patient prognosis, while low *PD-1* expression levels were correlated with poor prognosis [36]. More interestingly, Carreras *et al*. found that higher *PD-1*-positive cells in follicular lymphoma led to higher 5-year overall survival and progression-free survival [37]. The *PD-1* expression on different cells with different contributions to the anticancer activity (T-cells, natural killer cells, and macrophages) in the tumor microenvironment is one of the major reasons justifying the complex relationship between *PD-1* expression in the tumor environment and prognosis [30].

The detection of markers for prognostic

evaluations and prediction of responses to treatment or remission rates are important for developing a suitable treatment plan in a patient's initial diagnosis of AML. Based on our study, *PD-1* can be suggested as a potential prognostic marker in AML. However, the exact role of *PD-1* in AML prognosis is still unclear and must be elucidated in further studies. Also, it was found that higher *PD-1* expression was associated with unfavorable courses of the disease. Recent research has found that in addition to immune system cells, myeloid leukemic cells could express costimulatory molecules such as *ICOS* and *PD-1*, which can thwart an effective anti-leukemic T-cell response [38].

In this context, the expression of B7 superfamily costimulatory molecules B7-2 (CD86) and B7-H2 (ICOSL) is important. The CD28/cytotoxic T lymphocyte antigen 4 (CTLA-4) family member inducible costimulatory *ICOS* is expressed on activated T lymphocytes. Furthermore, the existence of $B7\text{-}2^{+}$ and/or $B7\text{-}H2^{+}$ AML cell subpopulations has been linked to poor clinical outcomes such as hyperleukocytosis and limited disease-free or relapse-free survival [18, 38, 39]. Dolen *et al*. indicated that the increased expression of B7 family molecules like B7-H1 (PD-L1) and B7-DC (PD-L2) on AML cells, as well as downregulation of B7-H2 (ICOSL) on AML cells, which is linked to the immunosuppressive phenotype of AML cells, attenuation of helper T-cell responses, and promotion of regulatory T-cell differentiation, mainly through the PD1 pathway [20, 40]. In addition to immune system cells, PB mononuclear cells make up a portion of leukemic blasts; therefore, alterations in CD28 and CTLA-4 gene expression could also be attributed to AML blasts. The present study had some limitations. For example, the present study did not assess prognostic parameters like overall survival. Furthermore, follow-up time (short or long) should be considered in the interpretation of the findings of different studies. Also, only the response to induction therapy was assessed in this study, while studies of consolidation therapy with extended follow-up times may reveal different results. Therefore, future studies with extended follow-up times are needed to explore the relationship between *PD-1* expression and overall survival or relapse rate.

Conclusion

Our study revealed that *PD-1* and *ICOS* expression before induction therapy could be associated with patients' response to induction therapy. Indeed, low *PD-1* and *ICOS* expression levels before induction therapy were associated with favorable responses.

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Conflict of Interest

The authors had no conflict of interest to declare.

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