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The Role of Immune Check Point Gene Expression of Pd-1 and Tim-3 in Patients with Acute Myeloid Leukemia

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Abstract

Acute myeloid leukemia (AML) is a hematopoietic cell cancer that spreads quickly to the blood and rapidly developing in the bone marrow. The prognosis for patients with acute myeloid leukemia (AML) is still poor, despite recent improvements in the therapeutic landscape. In hematological malignancies, immune checkpoint inhibitors have been studied, such as AML; however, the role of program cell death -1(PD-1) and T-cell immunoglobulin and mucin domain 3 (TIM3) in AML has not been thoroughly elucidated yet. Thus, the current study conducted to investigate the PD-1 and TIM−3 gene expression in the AML patients and determine its associations with clinical outcomes and prognostic variables. The study collected 80 blood samples from acute myeloid leukemia (AML) patients and 40 blood samples from volunteer healthy individual were evaluated as control and real time quantitative (qRT-PCR) analysis was detect to performed PD-1 and TIM−3 expression. The result showed there was non-significant (P>0.0001) in expression of TIM-3 in patients with AML, while expression of PD-1 statistically has high significant difference (P≤0.0001). A cutoff value of PD-1 for patients vs. control was (0.853) with high sensitivity than cutoff value of TIM-3 for patients vs. control that can be diagnostically significant in distinguishing between patients and controls. Our data result showed that high expression of PD-1 in T cell is extremely correlated with progression of disease and down regulated gene expression of TIM-3 in AML patients.

Keywords: Acute myeloid leukemia, T-cell immunoglobulin and mucin domain 3, Program cell death -1and Real time quantitative (qRT-PCR).

الخلاصة

ابيضاض الدم النخاعي الحاد AMLهو سرطان الخلايا المكونة للدم الذي ينتشر بسرعة في الدم وينمو بسرعة في نخاع العظام. لا يزال تشخيص المرضى المصابين بسرطان الدم النخاعي الحاد AMLضعيفًا ، على الرغم من التطورات الأخيرة في المشهد العلاجي. تمت دراسة مثبطات نقطة فحص المناعة في أورام الدم الخبيثة ، مثل AML ؛ ومع ذلك ، فإن دور موت خلايا البرنامج PD-1والغلوبولين المناعي للخلايا التائية ومجال الميوسين 3 − TIM في AML لم يتم توضيحه بالكامل بعد. وهكذا ، أجريت الدراسة الحالية للتحقيق في التعبير الجيني PD-1 و TIM-3 في المرضى الذين يعانون من AML وتحديد ارتباطاته بالنتائج السريرية والمتغيرات الإنذارية. **الطريقة**: جمعت الدراسة ٨٠ عينة دم من مرضى ابيضاض الدم النخاعي الحاد AMLو ٤٠ عينة دم من متطوعين أصحاء تم تقييمها كعينة تحكم وتم إجراء تحليل qRT- PCR للكشف عن PD-1 و TIM − 3. **النتائج**: أظهرت النتائج عدم معنوية (P> 0.0001)حسب تعبير TIM-3 في مرضى AML بينما التعبير عن PD-1 له فرق معنوي كبير P ≤ (0.0001). كانت قيمة القطع لـ PD-1 للمرضى مقابل مجموعة التحكم (٠,٨٥٣) مع حساسية عالية من قيمة القطع لـ TIM-3 للمرضى مقابل مجموعة التحكم والتي يمكن تشخيصها ذات القيمة للتمييز بين المرضى والمجموعة التحكم. **الخلاصة**: أظهرت نتائج بياناتنا أن التعبير العالي عن PD-1 في الخلية التائية يرتبط ارتباطًا وثيقًا بتطور المرض وانخفاض التعبير الجيني المنظم لـ TIM-3 في مرضى AML.

INTRODUCTION

Hematopoiesis is known as the ability of self-renewing cells to produce mature blood cells [1]. In bone marrow, number of immature cells are increased, and occurrence of abnormalities in hematopoiesis is known as leukemia, which is a highly severe hematological malignancy [2][3]. The Iraqi Center of Hematology department in the Medical City of Baghdad recorded 3102 cases of leukemia, and this was in the period between January 2018 and December 2019. They also recorded 1402 cases in 2018 and 1700 in 2019 for

all other types of cancer [4]. Acute myeloid leukemia (AML) is a heterogeneous disorder of white blood cells, a clonal in the population of myeloid stem cells that starts to proliferate and differentiate from autonomous cellular controls. Therefore, the, peripheral blood, bone marrow and other tissues will contain many cancerous cells [5]. AML is a heterogeneous disorder of leukocytes in which, abnormal clonal populations of hematopoietic stem cells that differentiate and proliferate. Then, disrupting the healthy maturation of bone marrow hematopoietic stem cells.(myoblasts) resulting in production of white blood cells (leukocytosis), platelets (thrombocytopenia) , and decreases red blood cells (anemia), They are myeloblasts that are not functional [6]. Inhibitory checkpoints are molecules in the immune system that function to fine-tune or turn off an immune response. These molecules initiate intracellular signaling events that interrupt activation cascades, thereby leading to decreased T cell proliferation and cytokine production. This process is critical for the establishment and maintenance of peripheral tolerance during normal immune responses. [7].

According to studies on the immune microenvironment of AML, the immune system is controlled by leukemia cells through a dynamic process known as immunoediting. They do this by expressing ligands of the immune checkpoint receptors, which allows them to bypass the immune system's normal inhibitory checkpoints. This results in T-cell exhausted is a process that causes the immune system to be downregulated and T-cell function of gradual decline. This term may be used to explain how hematological and solid malignancies can escape the immune system [7][8].

Increased the expression of some inhibitory receptors such as programmed cell death protein 1, lymphocyte activation gene 3 and T-cell immunoglobulin mucin domain 3, is characteristic of T cells. [9] . TIM3 is a negative regulatory receptor found on CD8+, CD4+ T cells, dendritic cells, and T-regulatory cells. It functions to suppress Th1 responses. [9,10]. Tim-3 has been demonstrated to be a prognostic marker in a variety of solid tumors, and prognosis is negatively correlated with expression level [11][12].The Programmed death-1 Proteins (PD-1) which are present on the T and B cell surface which also promote self-tolerance and stimulate the immune system [13]. PD-1 is a key immune checkpoint that inhibits function of T cell after antigenic stimulation [14]. In conclusion, high expression of PD-1 in T cell is extremely correlated with progression of disease and down regulated gene expression of TIM-3 in AML patients. The aim of study to observe the role of pd-1 and tim-3 genes in AML patients by measuring the gene expression and their role of immune check point in prognostic variables of the disease.

MATERIALS AND METHODS

This study was carried out between September (2022) till March (2023). In the study, 80 AML-positive samples were included from individual patients of varying age groups (18-84). Patients categorized into the FAB according to AML Subtype including the FAB (M3), FAB (non- M3). The controls were forty samples from volunteers who appeared to be in good health. The samples were collected from both sexes of different age groups from Baghdad Teaching Hospital - Medical City. An automated blood count analyzer was used to obtain blood count indices at the time of sampling. All the study experiments were performed at Mustansiriyah University, College of Science, Department of Biology and accepted by the committee on scientific ethics. Ethical approval number BCSMU/1222/00023Z.

Exclusion criteria: Patients & control: Children less than 18 years old. Inclusion criteria: patients who were diagnosed with AML, Age≥18-year-old. No other acute diseases and malignance.

Genomic RNA Extraction from the blood sample

Total RNA was extracted from all samples using the TransZol Up Plus RNA Kit Reagent according to the manufacturer's instructions and the number of kits is ER 501-01. After the extraction Synthesis, the cDNA form mRNA was synthesized. cDNA synthesis of the first strand, a reaction component. Total RNA was reverse-transcribed to complementary DNA utilizing the cDNA Synthesis SuperMix and EasyScript® One-Step gDNA Removal Kit. (cDNA).

Quantitative Real Time PCR (qRT–PCR).

The expression levels of the TIM-3 and PD-1 genes were estimated by the reverse transcription-quantitative polymerase chain reaction (qRT-PCR) method, a sensitive technique for the quantifying of steady-state mRNA levels. To confirm the

expression of the target gene, a quantitative real-time qRT-PCR SYBR Green assay was used. Alpha DNA Ltd. (Canada) designed and synthesized primer sequences for the TIM-3 and PD-1 genes, then lyophilized and stored at -20°C.The PD-1and TIM-3 gene levels were normalized by amplifying the levels of the endogenous control gene GAPDH mRNA.

Analysis of data

1. Analyzing the data produced by the Software from the Rotor-Gene Q Series, which included:
   a. Each amplification reaction's CT values are noted.
   b. The amplification of the plots.
   c. The dissociation of the curves.
2. According to their equation of Livak ΔCT were determined. Unknown samples' expression levels were analyzed and normalized. by 2-ΔΔCT method where ΔΔCT (Ct target gene-Ct GAPDH) target (Tim-3and PD-1) genes [15].

Statistical Analysis

By using GraphPad Prism 7.0 statistical analysis was done to know and identify impacts of various parameters in the study and discrete variables are presented utilizing percentages with number, Mann-Whitney test were utilized. When comparing differences between two groups, there was a Mann-Whitney U-test applied. Calculating the probability for variables with a normal distribution ANOVA utilized one method for their analysis. The level of gene expression in patients and controls was examined using the receiver operator curve (ROC), and (P 0.05) was taken into consideration to be significant statistically.

RESULTS AND DISCUSSION

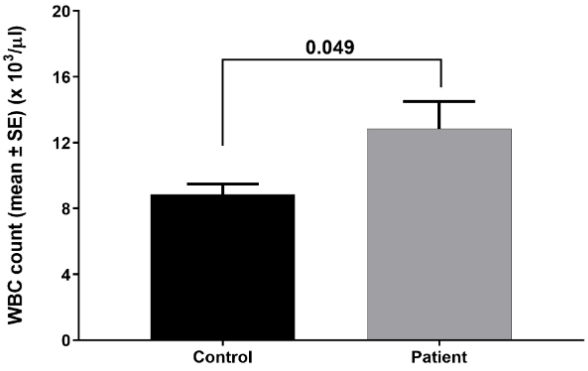
This study enrolled 80 participants and categorized them into two age groups. The first age group (≤ 40 years) had 38 (47.5) individuals in the group of patients and 20 (50) in the group of control individuals. The second age group (> 40 years) of 42 (52.5) individuals in the group of patients and 20 (50) individuals in the group of control. The P-value for all age categories was more significant than 0.05. The mean ± SE (mg/dL) of the patients' group (42.83 ± 19.08), while the mean ± SE (mg/dL) of the control was (40.4± 13.3), With the P-value higher than 0.05. there are no significant differences statistically were found between the patient groups. There was no discernible age difference between the group of control on one side and the various groups of AML patients on the other side (P = 0.626). Increased exposure to carcinogens, particularly environmental pollutions resulted in cancer appearing in younger age groups than originally present. This variation can be explained by the geographic and genetic differences between the two racial groupings, as well as by the higher mean ages in western countries than in the east [16].

A complete blood count was used to determine the following patients' characteristics: The patients and the control median white blood cell counts (10^3/L) were (8.55×10^3) and (5.99×10^3) respectively, and they demonstrated significant differences between each group under study, as shown in Figure 1(a).

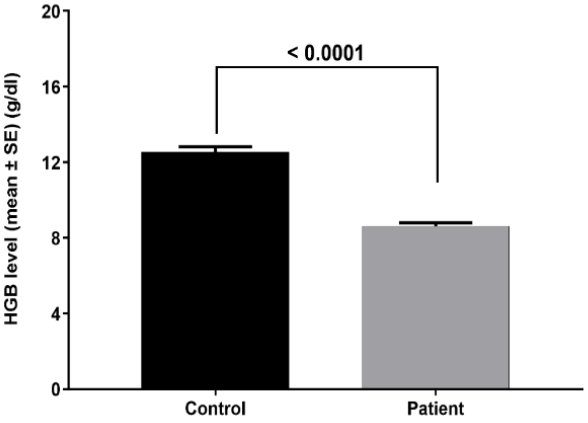
For the patients and the control group, the hemoglobin level median was (12. 85 g/dl), (8.25 g/dl) respectively, according to the hemoglobin level between the group of patients and the group of control there was a significant difference (P ≤0.0001) as show in Figure 1(b). The platelets count ×103/μL in both the patients and control groups median was (230.5×103), (59.75×103), respectively that showed significant difference, as shown in Figure 1(c). The result of the current study also agreed with the study that was done by Ahmed, who deduced a significant difference existed between controls and AML patients. (P=<0.001) in the CBC parameters like platelets and hemoglobin, while the result was contradictory for the WBC count [17]. The level of platelets in the current study had high significant differences between patients and control subjects, respectively, while there were without significant differences between the types of patients who were suffering from thrombocytopenia. Thrombocytopenia was common in patients as a result of taking chemotherapy, so the low platelet count may be the cause of bleeding in AML patients.

Quantitative real time polymerase chain reaction qRT-PCR analysis of PD-1 and TIM-3 transcripts and peripheral blood indices, all AML cases were classified based on diagnosis. there was a significant difference (P=<0.0001), The median expression of PD1 was 1.057 in the AML patients, while in the healthy controls was 0.561. The current study results show markedly increased expression

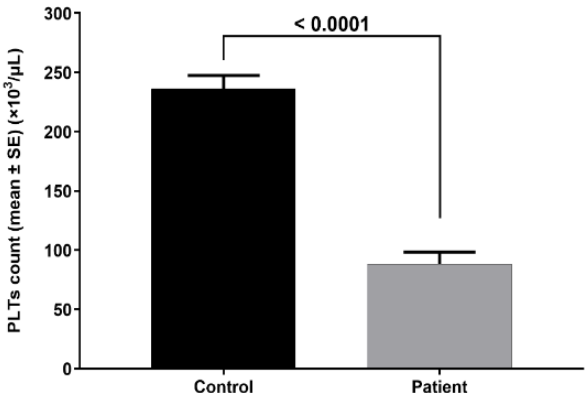
of PD-1 in AML patients, and are highly significant compared to those in healthy controls. (P<0.0001) as shown in Figure 2. This result corresponds with Ruan who observed that the expression of PD1 was increased significantly and the levels of PD1 in AML (p<0.0001), and AML (p<0.0001) were obviously higher than those in controls [18]. The high expression of PD-1 the activates of PD-1/PD-L1 signaling pathway continuously and inhibits the various signaling pathways [16][17].



(a) *P≤0.05



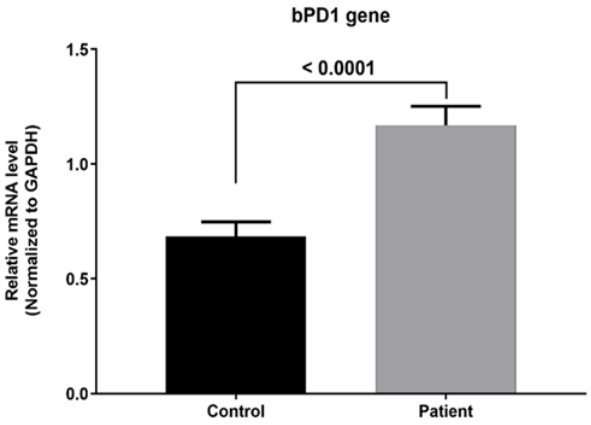
(b) **P≤0.01



(c) **P≤0.01

Figure 1. Characteristics of the patients based on complete blood count between control group and AML groups. (a) white blood cells count (b) hemoglobin level (c) Platelet count.

The tim-3 expression in the patients AML was 0.00018 while in the healthy controls was0.000269 and the P-value is 0.6325.TIM-3 expression was downregulated significantly as shown in Figure 3 TIM-3 is one of the immunoregulatory proteins in the TIM family.

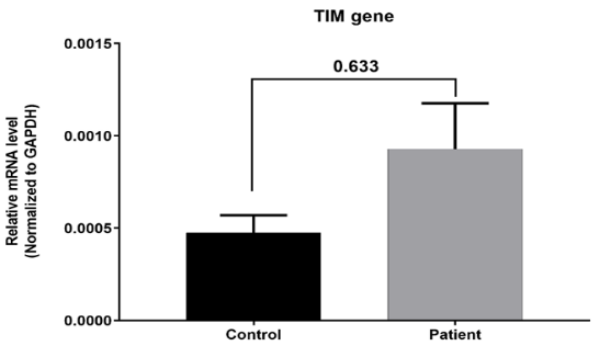


****P≤0.01**

Figure 2. Shown P-value of PD-1expression level in patient and control.

It has been considered an insignificant checkpoint receptor since its initial discovery in T cells [19]. Although, in the immune suppression of tumors, tim-3 is a crucial immune checkpoint, its function in AML is still unknown [20]. Therefore, the current study examined expression of Tim-3 in AML patients and evaluated its significance in clinical as a possible prognostic tool for AML adults. The results showed expression of tim-3 was downregulated significantly in AML patients compared to the control group by RT-qPCR analysis.

Similar to our results that show the expression of TIM-3 levels in leukemic blasts not significantly correlated with event-free survival (p=0.9873) or the status of remission after chemotherapy induction (p=0.9799) [21].



P≥0.05NS: non-Significant

Figure 3. shows P-value of TIM-3expression level in patients and control.

In addition, as shown in Table 1, the analysis of ROC curves was performed to find the value of the optimum cut-off of PD-1 and TIM-3 expression to differentiate between AML patients and healthy control. The results showed mild diagnostic value with the value of AUC 0.725,0.527, respectively and sensitivity 66.25, 56.25 and specificity were 67.5 and 57.5% respectively, as shown in Figures 4 and 5.

Table1. The analysis of ROC for PD-1 and TIM-3 expression

Comparison groups	control vs. patients of pd-1	control vs. patients of tim-3
AUC	0.725	0.527
95% CI of AUC	0.632-0.818	0.418-0.636
P-value	<0.0001	<0.63
SN (%)	66.25	56.25
SP (%)	67.5	57.5

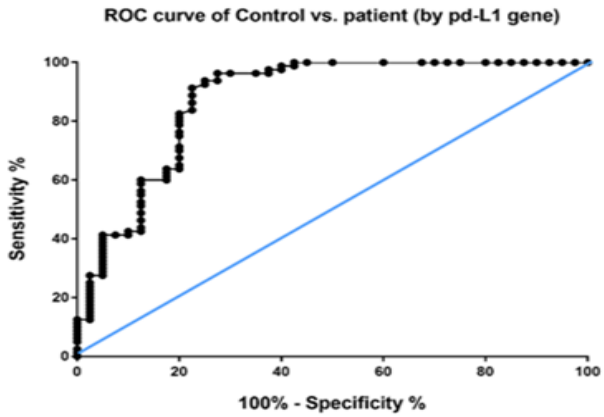


Figure 4. ROC curve for expression PD-1 for control vs. patients.

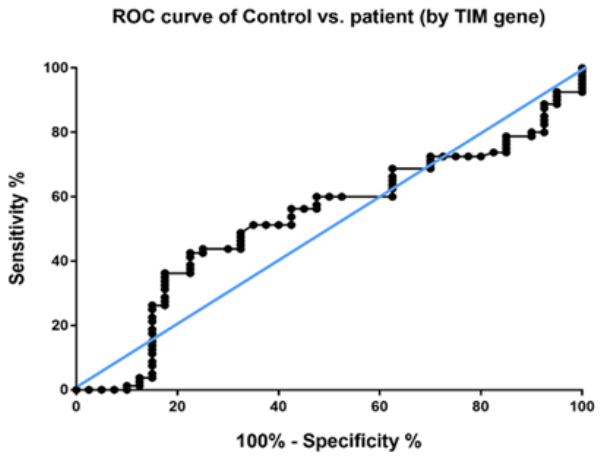


Figure 5. ROC curve for expression tim-3 for control vs. patients.

Despite in the fact that Tim-3 expression on various T-cell subsets could negatively regulates the antitumor immunity in patients with cancer. New treatment targeting TIM-3 and PD-1. Tim-3 and other negative checkpoint regulators on T cells, Tim-3 on NK cells and LSCs, and combined chemotherapy or cancer vaccines with mAb to Tim-3 or Tim-4 may provide a breakthrough in the treatment. Further studies to investigate the role of genes in AML chemotherapy treatment.

CONCLUSIONS

These conclusions of the current study showed that PD-1 expression was upregulated significantly in AML patients. In addition, the expression of TIM-3 was downregulated, suggesting that TIM-3 and PD-1 can serve as a biomarker for a poor prognosis in AML. However, required Studies further are needed to confirm using PD-1 and TIM-3 as potential AML therapeutic targets.

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Disclosure and Conflict of Interest: The authors declare that they have no conflicts of interest.

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