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THE ROLE GENE EXPRESSION OF PD-1 AND PD-L1 IN NEWELY DIAGNOSED AND TREATED PATIENTS WITH ACUTE MYELOID LEUKEMIA

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Abstract.

Acute myeloid leukemia (AML) is a myeloid malignancy in which hematopoietic progenitor cells are blocked early in development, causing the development of abnormal cells. The most common type of adult leukemia is AML. The most significant developments in the treatment of cancer over the past ten years have been made possible by programmed death protein 1 and anti-programmed ligand 1 (PD-L1) which are examples of immune checkpoint (IC) inhibitors. This study involved two groups: the patient group, consisting of 80 samples, and the control group, consisting of 40 samples. The participants' age range was 18-85 years, and the samples were obtained from at Baghdad Teaching Hospital - Medical City in Baghdad, Iraq. Patients were categorized into the FAB according to AML Subtype including the FAB (M3), FAB (non- M3). The age group did not show a significant difference (P≥0.05) in patients with AML compared to the control group. Furthermore, The mean age of patients was 42.83 years, and control age mean 40.4 years.

The aim of this study was to evaluate the effect of Acute myeloid leukemia on the levels of certain immunological parameters. The results of the QRT-PCR technique for immunological tests showed the PD-1 expression in patients with AML statistically has high significant difference (P ≤ 0.0001), and the PD-L1 expression also had a highly significant difference (P < 0.0001) in PD-1, PD-L1 genes, compared to the control group. These findings suggest that AML infection may influence immunological responses.

Key words. Acute Myeloid Leukemia, Program Cell Death-1, Program Cell Death Ligand-1.

Introduction.

Hematopoiesis is the ability of self-renewing cells to produce mature blood cells [1]. In bone marrow the number of immature cells are increased, and occurrence abnormalities in hematopoiesis are known as leukemia, which is a highly severe form of hematological malignancy [2]. 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 [3]. Acute myeloid leukemia (AML) is a malignant, aggressive hematological condition having a low prognosis and a high mortality rate [4]. It is the most typical form of adult acute leukemia [5]. Programmed death-1 Proteins (PD-1) are present on the T cell and B cell surfaces and in addition, it is known CD279 (Cluster of Differentiation 279) which also promote self-tolerance, stimulate immune system [6], PD-1 is a key immune checkpoint that inhibits function of T cell after antigenic stimulation [7]. The PD-L1 ligand is referred to as programmed cell death ligand-1 (PD-L1) and produced by tissue cells, like cancer cells and the antigen-presenting cells [8,9].

In general, the PD-1/PD-L1 inhibitor checkpoint interacts with the ligand PD-L1 to prevent CD8+ T cell proliferation and T cell receptor-mediated cytotoxicity. As a result, immune surveillance destroys the cancer cells, and the autoimmune system is prevented from attacking tumor cells [10,11]. In AML, the PD-1/PD-L1 pathway is hijacked by malignant cells to facilitate immune escape. Many preclinical studies have demonstrated up-regulation of the PD-1/PD-L1 pathway in AML and the negative impact of this amplification on disease control. However, the clinical response to PD-1/PD-L1 blockade varied in different AML patients [12]. A recent study revealed that the majority of immune-checkpoint receptor genes were downregulated in bone marrow (BM)-infiltrating CD8+ T cells and partially in CD4+ T cells due to pathological chromatin remodeling via histone deacetylation. Therefore, the dysfunction of CD8+ T cells in AML was mainly due to pathological epigenetic silencing of activated IC receptors rather than due to signaling by immune inhibitory IC receptors [13]. This may explain the limited role of PD-1/PD-L1 antibodies in AML patients. In conclusion, anti-PD-1/PD-L1 therapy may be a new immunotherapeutic strategy for AML. However, further studies are still necessary.

The aim of study observing the role of PD-1 and PD-L1 genes in AML patients by measuring the gene expression and their role of immune checkpoint in prognostic variables of the disease.

Materials and Methods.

The study was conducted between September 2022 and March 2023 at Baghdad Teaching Hospital - Medical City in Baghdad, Iraq. The study involved two groups: the patient group and the control group.

The patient group consisted of 80 samples collected from individuals who were presented with AML-positive. The control group volunteers who appeared to be in good health, on the other hand, consisted of 40 samples. Both male and female participants from various age groups (18 to 85 years) were included in the study.

Exclusion criteria: Patients & control: children less than 18 years old, chronic disease and malignance. Inclusion criteria: Persons who have been diagnosed with AML, Age ≥18 year – old.

Blood samples and measurement of genes parameters:

Blood samples were obtained from both the patient and control groups using disposable syringes. A total of 5 ml of blood was collected into an ethylene diamine tetra acetic acid (EDTA) tube, then dispensed into an Eppendorf tube which contains the TRIzol in Eppendorf mixing with 250 microliters of blood. The collected blood in the Eppendorf tube was then stored at -20°C until further use.

To measure the Eppendorf tube blood's RNA was extracted to determine the PD-1 and PD-L1 specific kits (Trans Gen biotech,
China). The qRT-PCR assays were performed following the manufacturer's instructions, adhering to the prescribed protocols and procedures.

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:
   \[ \Delta C_T (\text{patient}) = C_T (\text{gene}) - C_T (\text{H.K.G}) - \Delta C_T (\text{control}) = C_T (\text{gene}) - C_T (\text{H.K.G}) \]
   By subtracting the Ct value of each test group from the Ct value of the control group, we were able to get the Double Delta Ct Value (Ct) for the genes of interest. At last, the formula was used to determine the expression ratio:
   \[ \Delta \Delta C_T = \Delta C_T (\text{patient}) - \Delta C_T (\text{control}) [14]. \]

Statistical Analysis:

By using GraphPad Prism 7.0 statistical analysis was done to know and detect the effect of various parameters in study, discrete variables presented using their percentage and number, Mann-Whitney test were used. When comparing differences between two groups, the Mann-Whitney U test was used. 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 statistically significant.

Results and Discussion.

Our current research included 80 patients with AML and 40 healthy individuals, with samples collected from the Medical City, Baghdad Teaching Hospital, hematology department. This study categorized participants into four age groups. The first age group (≤ 20 years) had 1 (2.5%) individuals in the control group and 11 (13.75%) individuals in the patient group. The second age group (21-40 years) consisted of 19 (47.5%) individuals in the control group and 27 (33.75%) individuals in the patient group. The third age group (41-60 years) included 17 (42.5%) individuals in the control group and 27 (33.75%) individuals in the patient group. The fourth age group (> 60 years) comprised 3 (7.5%) individuals in the control group and 15 (18.75%) individuals in the patient group. The P-value for all age categories was greater than 0.05. The P-value for all age categories was greater than 0.05.

The mean ± SE (mg/dL) of the control group was (40.4 ± 13.3), while the mean ± SE (mg/dL) of the patient group was (42.83 ± 19.08), with a P-value greater than 0.05, as shown in Table 1.

The study found no significant correlation between age and AML. The mean age of patients was 42.83 years and the control age mean 40.4 years. which is near to results reported by Sultan, the median age of the patients in their study was 34.5 years [15]. When compared with international reports, our finding is in distinction with studies published from Germany and Sweden where the median age was 60 and 71 years respectively [16,17]. However, certain studies from our part earlier reported low median age [18]. We found a significant positive correlation between PD-1/PD-L1 expression with various age (r= 0.186).

### Table 1. Distribution of samples is categorized according to age in both the control and patients’ groups.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Control without AML / No (%)</th>
<th>Patients infected with AMLs / No (%)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Average age (years ± SE)</td>
<td>40.4 ± 13.3</td>
<td>42.83 ± 19.08</td>
<td>0.626NS</td>
</tr>
<tr>
<td>≤ 20</td>
<td>1 (2.5%)</td>
<td>11 (13.75%)</td>
<td>0.06</td>
</tr>
<tr>
<td>21-40</td>
<td>19 (47.5%)</td>
<td>27 (33.75%)</td>
<td></td>
</tr>
<tr>
<td>41-60</td>
<td>17 (42.5%)</td>
<td>27 (33.75%)</td>
<td></td>
</tr>
<tr>
<td>&gt; 60</td>
<td>3 (7.5%)</td>
<td>15 (18.75%)</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>40</td>
<td>80</td>
<td></td>
</tr>
<tr>
<td>NS: Non-Significant.</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

### Table 2. Distribution of study samples according to gender in the control and patients’ groups.

<table>
<thead>
<tr>
<th>Group No</th>
<th>Gender</th>
<th>Male No. (%)</th>
<th>Female No. (%)</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>40</td>
<td>24 (60)</td>
<td>16 (40)</td>
<td>0.3006</td>
</tr>
<tr>
<td>Patients</td>
<td>80</td>
<td>40 (50)</td>
<td>40 (50)</td>
<td></td>
</tr>
</tbody>
</table>

### Table 3. Distribution of samples is categorized according to AML subtypes in both the control and patients’ groups.

<table>
<thead>
<tr>
<th>FAB classification</th>
<th>Number</th>
<th>Percentage</th>
</tr>
</thead>
<tbody>
<tr>
<td>M1</td>
<td>5</td>
<td>6.25%</td>
</tr>
<tr>
<td>M2</td>
<td>1</td>
<td>1.25%</td>
</tr>
<tr>
<td>M3</td>
<td>25</td>
<td>31.25%</td>
</tr>
<tr>
<td>M4</td>
<td>3</td>
<td>3.75%</td>
</tr>
<tr>
<td>M5</td>
<td>7</td>
<td>8.75%</td>
</tr>
<tr>
<td>Unknown</td>
<td>39</td>
<td>48.75%</td>
</tr>
</tbody>
</table>

This variation can be explained by the geographic, or habits of dietary and genetic differences between the two racial groupings, as well as by the higher mean ages in western countries than in the east. Increased exposure to carcinogens, particularly environmental pollutions, resulted in cancer appearing in younger age groups than originally present.

Table 2 shows the distribution of groups according to gender revealing an approximately numerical ratio between females and males in both healthy control and patients, respectively. The percentage of males in the healthy control group was (60%), while the percentage of patients was (50%). The percentage of females in the control group was (40%). The percentage of females was in patients (50%). There was a significant difference between control and patients with a p-value of 0.3006.

Males and females both there are affected with the disease, according to the current study, this was probably because the nature of environment or exposure to the radiation and also possibly due to mutations of the Tumor protein 53 (TP53) tumor suppressor gene, which are more common in males. These results are contrast to the results of Alwan et al which showed that males are more susceptible to disease compared to females [19].

Table 3 shows the percentages of AML subtypes. The groups in this study were divided into five subtypes of AML to account for the subjective differences in AML subgroups based on morphologic diagnosis and the variable nature of AML. FAB (M3) that are most common in AML (31.25%), This subtype is classified separately because its treatment strategy is different.
from the other FAB subtypes. The current study shows that there are multiple subtypes, with M5 being the most common (8.75%), followed by M1 (6.25%), M4 (3.75%), and M2 (1.25%). M0 and M6 are very rare according to the current study.

The result showed that the most common type is (M3). The result of the current study in case of FAB (M3) was agree with the results of previous studies such as the study of Bashasha, where the percentage was 28.5% and 26.9%, respectively [20]. Asif and Hassan showed that M1 was the common subtype followed by M3 and M4 at the same level, and this result was in contrast to the current study, which showed that M3 is the most common [21]. Arber found that M2 was the most common, followed by M5, while their percentage in the current study was 1.25% and 8.75%, respectively [22].

The further study observed that a complete blood count was used to determine the following patient characteristics: The patients and the control median white blood cell counts (10³/L) were (8.55×10³) and (5.99×10³) respectively, and they demonstrated significant differences between each group under study. For the patients and control group, the median hemoglobin level was (12.85 g/dl), (8.25 g/dl) respectively, according to the hemoglobin level between the patients group and the control group there was a significant difference (P ≤0.0001). The median platelets count ×10³/µL in both the patient and control groups was (230.5×10³), (59.75 ×10³) respectively that showed significant difference, as shown in table 4.

The level of platelets in the current study had high significant differences between patients and apparently healthy 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. The current study was consistent with the study conducted by Asif and Hassan in which the majority of patients had a platelet count of less than 50 [21].

The gene expression of PD-1 and PDL-1 in the Study Groups

The recent study revealed 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 based on table 3 and are highly significant compared to that in the healthy control (P < 0.0001) as shown in Figure 1. Additionally, the median of PD-L1 expression was 1.79 in the AML patients while in the healthy controls was 0.247. Based on the table 3. The current study results showed a significant increase PD-L1 expression in AML patients than that in the healthy control (P < 0.0001) as shown in Figure 2.

Our study showed the median expression of PD1 was 1.057 in the AML patients, while in the healthy controls was 0.561.

### Table 4. Patient and control comparison in CBC parameter.

<table>
<thead>
<tr>
<th>Group</th>
<th>WBCs count median (×10³/µL) (range)</th>
<th>HGB level median (g/dl) (range)</th>
<th>PLTs count median (×10³/µL) (range)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>8.55 (2.2-31.7)</td>
<td>12.85 (6.3-14.6)</td>
<td>230.5 (3-393)</td>
</tr>
<tr>
<td>Patients</td>
<td>5.99 (0.13-45)</td>
<td>8.25 (4.03-16.42)</td>
<td>59.75 (3-420)</td>
</tr>
<tr>
<td>P-value</td>
<td>0.049*</td>
<td>&lt;0.0001**</td>
<td>&lt;0.0001**</td>
</tr>
</tbody>
</table>

*P≤0.05, **P≤0.01

### Table 5. ROC analysis is used to PD-1/PD-L1 expression.

<table>
<thead>
<tr>
<th>Comparison groups</th>
<th>AUC</th>
<th>95% CI of AUC</th>
<th>P-value</th>
<th>Optimum Cut off value</th>
<th>SN (%)</th>
<th>SP (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control vs. Patient of PD-1</td>
<td>0.725</td>
<td>0.632-0.81</td>
<td>&lt;0.0001</td>
<td>0.853</td>
<td>66.25</td>
<td>67.5</td>
</tr>
<tr>
<td>Control vs. Patient of PD-L1</td>
<td>0.875</td>
<td>0.799-0.951</td>
<td>&lt;0.0001</td>
<td>0.622</td>
<td>91.25</td>
<td>77.5</td>
</tr>
</tbody>
</table>

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

Figure 2. Shown P-value PD-L1 expression level in patient and control.

Significant variations between control and the patient were seen in white blood cell counts because the patients initial stages of chemotherapy. The median hemoglobin level when comparing the patient groups to the control was significantly different (P 0.0001), and the patient groups had lower hemoglobin levels than the control group. The result of the current study also concurred with the research conducted by Ahmed, who deduced were differences significant between AML patients and controls (P=0.001) in platelets and hemoglobin [23].

The gene expression of PD-1 and PD-L1 in the Study Groups

The recent study revealed 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 based on table 3 and are highly significant compared to that in the healthy control (P < 0.0001) as shown in Figure 1. Additionally, the median of PD-L1 expression was 1.79 in the AML patients while in the healthy controls was 0.247. Based on the table 3. The current study results showed a significant increase PD-L1 expression in AML patients than that in the healthy control (P < 0.0001) as shown in Figure 2.

Our study showed the median expression of PD1 was 1.057 in the AML patients, while in the healthy controls was 0.561.
Level expression of PD-1 in AML patients is highly significant compared to that in the healthy control (P < 0.0001). This result corresponds with Ruan who observed that the expression of PD1 was significantly increased and the levels of PD1 in AML (p<0.0001), and AML (p<0.0001) were obviously elevated than those in controls [24]. The median of PD-L1 expression was 1.79 in the AML patients while in the healthy controls was 0.247 with level expression of PD-L1 in AML patients than that in the healthy control (P<0.0001), which is near to results reported by mostafa et al. who demonstrated that PD-L1 expression in their patients ranged from 1.52% to 88.1% [25]. The main aim of our study is to observe and identify the relationship between PD-1 and PD-L1 expression in patients with AML and different disease characteristics to evaluate the pathway of immunosuppressant. A wide variety of solid tumors and blood malignancies are being successfully treated with antibody-based PD-1/PD-L1 blocking treatments. This form of therapy can reverse the immunosuppressive environment in the BM and restore CD8+ T cell antitumor responses [26].

We found in our study a significant positive correlation between PD-1 and PD-L1 expression (r=0.340) and the expression values were significantly upregulated in the patients with AML. Zajac et al. showed that PD-L1 expression is associated with unfavourable clinical outcome in AML patients [27]. Also, Annibali et al. concluded that the existence of PD-L1 on AML blasts was associated with negative course of the disease [28]. We support that level of PD-1 and PD-L1 expression affect the outcome after induction therapy in AML patients and it is possible to rely on this level expression to diagnosis and the prognosis of the disease.

The ROC analysis is used to PD-1/PD-L1 expression.

The study revealed A receiver operating characteristic (ROC) curve analysis was used to establish a cutoff value for pd-1 expression levels at (0.853), as can be seen in Table 5 with The AUC was 0.725, the diagnostic sensitivity and specificity were 66.25 and 67.5%, respectively as shown in Figure 3.

Conclusion.

Our data results show that PD-1and PD-L1 is expressed in AML patients with upregulated levels. PD-1/PD-L1 blockade has been proved to be effective in AML patients. Understanding as much as possible about the PD-1/PD-L1 pathway in AML is important and has a significant effect on treatment planning, toxicity management, and patient selection.

The analysis indicated that there was no significant difference in the age distribution between patients infected with the Acute myeloid leukemia and the control group.

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Conflicts of Interest: No conflict of interest as authors declares.

Ethical approval: Mustansiriyah University oversaw and approved this study by the Ethics Committee.

REFERENCES


