Estimation of Serum TLR-9, TNF-α, and IL-6 Levels in the Iraqi Patients Diagnosed as Acute Myelogenous Leukemia

Maryam Qasim Mohammed⁠¹, Ali Hussein Alwan⁠², Asmaa Amer Almukhtar⁠³

¹Department of Biology, College of Science, Mustansiriyah University, Baghdad, Iraq.
²Iraq center for cancer and medical genetics research, Mustansiriyah university, Baghdad, Iraq.
³Department of Medical Genetics, Iraqi Center for Cancer and Medical Genetics Research, Mustansiriyah University, Baghdad, Iraq.

*Corresponding Author.

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Abstract

Acute myelogenous leukemia (AML) is a varied group of biological and clinical diseases. It is distinguished by the fast proliferation of aberrant cells in the bone marrow, which interferes with normal blood cell formation. Several studies demonstrated that cytokines released by leukemic cells in an autocrine or paracrine manner influence the proliferation of AML cells. Our study aimed to assess serum levels of TLR-9, TNF-α and, IL-6 in patients of AML. The study was done via the ELISA Technique. The results show a highly significant difference in all parameters in Patients and control. The correlation between immunological parameters using the Pearson correlation coefficient for patients showed that there is a weak correlation between TLR-9 and TNF-α, IL-6 with P-values of 0.47 and 0.23 respectively. This may indicate the complex environment of inflammation. However, there was a strong correlation between TNF-α and IL-6 with a P-value (0.001). This indicates that the secretion of these cytokines was high in patients. All immunological parameters of patients were evaluated using the receiver operating characteristic (ROC) curve to demonstrate that any one of them is a useful tool for identifying and tracking inflammation. The ROC curve's findings revealed that all parameters have good sensitivity and specificity for detecting inflammation and disease activity in AML patients. According to the current study, AML was related to higher levels of TLR-9, TNF-α, and, IL-6 may be affected the prognosis of the disease, increase the risk of the disease progression, and may be used as a biomarker to target AML cells.

Keywords: Acute Myeloid Leukemia (AML), Toll-Like Receptor-9 (TLR-9), Interleukin-6 (IL-6), Tumor Necrosis Factor Alpha (TNF-α), Tumor Microenvironment (TME), Acute Promyelocytic Leukemia (APL).

Introduction

Acute myelogenous leukemia (AML) is a diverse collection of illnesses in terms of Biology and clinical development. It is distinguished by the fast propagation of aberrant cells found in the bone marrow, which interferes with the normal blood cells' development, the most commonly diagnosed leukemia in adults (25%) and comprises 15–20% in children.¹²³ Leukemic blasts and immune system cells can both produce cytokines in AML patients, although it is unclear what function they play in the etiology of the disease and their role in the pathophysiology of acute leukemia is not entirely understood. Therefore, leukemia is characterized by aberrant cytokine signaling that may contribute to
propagation, blast persistence, patient diagnosis, and treatment resistance. In addition, the immunological microenvironment is a crucial regulator and driver of the development of leukemia and hematological disease.

The aberrant cytokine signaling is a leukemia feature that may contribute to proliferation, blast survival, therapy resistance, and patient prognosis, leukemic blasts from many AML patients, unlike normal hematopoietic cells, release cytokines such as IL-1, GM-CSF, G-CSF, IL-6, IL-8, TNF-α, and SCF.

One of the primary reasons for the growth of cancer cells is the interaction between inflammation and the tumor microenvironment (TME). TME is a complex network involving tumor and immune cells, cytokines, enzymes, and other components. Pro-inflammatory cytokines, particularly interleukin-6 (IL-6) and tumor necrosis factor-alpha (TNF-α) play an important role in the development of chronic inflammation in the tumor environment. TNF-α is an inflammatory cytokine primarily produced by macrophages, able to promote cell differentiation and proliferation as well as cell death and regeneration so it has been linked to a variety of illnesses including cancer. This cytokine plays an essential role in cancer formation due to involved in all cellular stages including survival, proliferation, transformation, angiogenesis, invasion, and metastasis.

The TNF-α gene is found on chromosome 6p21.3 and is mostly expressed by activated macrophages, NK cells, and T lymphocytes; however, it has also been found in fibroblasts, astrocytes, Kupffer cells, smooth muscle cells, keratinocytes, and tumor cells. TNF-α has a role in leukemogenesis at every stage, including cellular change, proliferation, extramedullary infiltration, and angiogenesis. It is also a key player in the tumor microenvironment, assisting leukemia cells in immune Evasion, treatment resistance, and, survival, and maybe a promising marker for leukemia treatment. It is a key inflammatory regulator that has been shown to upregulate molecules involved in the growth and propagation of cells in tumors via NF-KB both dependent and independent pathways.

Interleukin-6 (IL-6) is a multifunctional interleukin that plays an important role in immune response, inflammation, and hematopoiesis in response to injury or infection. A variety of cells produce IL-6 in response to various stimuli, including in response to infection and diseases like cancer where numerous types of cancer cells were shown to have greater IL-6 levels than normal cells, while demonstrating the role of IL-6 in the etiology of many diseases, including multiple myeloma, lymphoma, acute myelogenous leukemia, and it may be a prognostic factor for solid tumors, such as prostate cancer.

Through the IL-6/IL-6 receptor signaling arrangement, IL-6 is a critical prognostic factor in chronic lymphocytic leukemia, and large cell lymphoma, and can contribute to the formation of AML blast cells, as well as stimulation and preservation of their growth, more aggressive leukemic phenotypes were correlated with overexpression of IL-6. Interleukin-6 levels in the blood can be employed as prognostic serum markers as well as follow-up criteria for early detection of relapse in people with acute myeloid leukemia. Cytokines have been shown to exert a significant influence on the progression of hematopoietic malignancies including AML such of these cytokines is IL-6 has functional pleiotropy and redundancy, generating responses from a variety of cell types.

Toll-like receptor (TLRs) stimulation results in the release of several pro-inflammatory cytokines and chemokines, which can stimulate tumorogenesis by promoting cell propagation and migration and developing a favorable microenvironment for tumor cells. Overexpression of several TLRs has been associated with tumor cell survival, proliferation, and metastasis in a variety of cancers, including colon, breast, and lung cancers. TLRs are expressed or up-regulated in tumors and tumor cell lines, but their level of expression and function in the etiology and development of acute leukemia in children has established little concern. Due to that TLRs can indirectly promote tumor development by aiding the formation of an inflammatory milieu through the production of cytokines, the current study aimed to evaluate the TLR-9 serum levels due to increasing their level in the responsiveness of human cells to CpG DNA that may support the hypothesis of immune escape by leukemic cells because leukemia is characterized by an impaired...
immune system. The pro-inflammatory cytokines like TNF-α, and IL-6 as one product of TLR-9 stimulation products in Iraqi patients diagnosed with acute myelogenous leukemia.

Materials and Methods

Patients and Control
A total of 90 samples were included in this study. 45 samples were from patients of AML, and 45 were from healthy controls both males and females with ages ranging from (15-83 years). Samples were collected during the period from October 2021 to January 2022 from Madinat Al-Tibb Hospital and Madinat al-Amamin Al Kadhim Hospital, Baghdad, Iraq. The AML patients were diverse including M2, M3 (acute promyelocytic leukemia), M4, M5, secondary AML, and relapsed according to flow cytometry results.

Methods
Serum levels of TLR-9, IL-6, and TNF-α were evaluated for both patients and healthy subjects using MyoBioSource Sandwich ELISA kits. Hematological parameters such as Total WBC, RBC, HGB, HCT, lymphocytes, and platelets were measured by complete blood count (CBC) according to Mustafa, (2019).^{18}

Statistical Analysis:
To determine the effect of numerous factors like hematological and immunological parameters in both groups of the current study including patients and control, the statistical analysis system- SAS (2018) program was utilized. In this investigation, the T-test was employed to compare between means. Pearson correlation coefficient test in the statistical package of social science (SPSS) was determined. 0.05and, 0.01 was probability in the current study.

Results and Discussion
To study the differences between the groups of this study represented by patients and healthy control, the Mean± standard error (SE) was used. The result of hemoglobin, white blood cells, lymphocytes, hematocrit, red blood cells, and platelets for both patients and control are shown in Table 1.

<table>
<thead>
<tr>
<th>Group</th>
<th>WBC (x 10^9 L)</th>
<th>Lymphocytes (x 10^9 mL)</th>
<th>Hb (g/dl)</th>
<th>PLT (x 10^9)</th>
<th>RBC (x 10^6 mL)</th>
<th>HCT (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>7.49 ±0.27</td>
<td>4.04 ±1.03</td>
<td>14.57±0.36</td>
<td>257.97±11.8</td>
<td>4.92 ±0.10</td>
<td>42.47±0.98</td>
</tr>
<tr>
<td>Patients</td>
<td>10.55 ±2.41</td>
<td>4.34 ±1.88</td>
<td>8.34 ±0.33</td>
<td>69.78±12.20</td>
<td>2.89 ±0.12</td>
<td>25.07±1.00</td>
</tr>
<tr>
<td>T-test</td>
<td>4.827 NS</td>
<td>4.906 NS</td>
<td>0.972 **</td>
<td>33.078 **</td>
<td>0.317 **</td>
<td>2.798 **</td>
</tr>
<tr>
<td>P-value</td>
<td>0.210</td>
<td>0.886</td>
<td>0.0001</td>
<td>0.0001</td>
<td>0.0001</td>
<td>0.0001</td>
</tr>
</tbody>
</table>

** (P≤0.01).

The current study showed a highly significant difference between patients and healthy control for serum IL-6, TNF-α, and, TLR-9 levels where Mean± SE for both patients and healthy control is shown in Table 2 with P-value (0.001) for IL-6, TNF-α, and TLR-9, respectively.

<table>
<thead>
<tr>
<th>Group</th>
<th>TLR-9</th>
<th>TNF-α</th>
<th>IL-6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>2.219 ±0.06</td>
<td>107.98 ±3.25</td>
<td>22.82 ±0.72</td>
</tr>
<tr>
<td>Patients</td>
<td>2.728 ±0.04</td>
<td>174.22 ±4.07</td>
<td>36.24 ±0.86</td>
</tr>
<tr>
<td>T-test</td>
<td>0.156 **</td>
<td>10.367 **</td>
<td>2.242 **</td>
</tr>
<tr>
<td>P-value</td>
<td>0.0001</td>
<td>0.0001</td>
<td>0.0001</td>
</tr>
</tbody>
</table>
The results of the correlation between the immunological parameters for this study using the Pearson correlation coefficient and p-value for patients are shown in Table 3. There is a strong correlation between TNF-α and IL-6 (r=0.788, P<0.001) indicating that the secretion of these cytokines was high in patients, while there is a weak correlation between TLR-9 and TNF-α, IL-6 with P-value (0.47 and 0.23 respectively), this may indicate the complex environment of inflammation. The result of the current study indicated the stimulation of the immune response due to the secretion of these cytokines.

### Table 3. Correlation between TLR-9, TNF-α, and IL-6 in patients.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Pearson Correlation</th>
<th>TLR-9</th>
<th>IL-6</th>
<th>TNF-α</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR-9</td>
<td>r</td>
<td>1</td>
<td>0.182</td>
<td>0.112</td>
</tr>
<tr>
<td></td>
<td>P-Value</td>
<td></td>
<td>0.23</td>
<td>0.47</td>
</tr>
<tr>
<td>IL-6</td>
<td>r</td>
<td>0.182</td>
<td>1</td>
<td>0.788**</td>
</tr>
<tr>
<td></td>
<td>P-Value</td>
<td>0.023</td>
<td></td>
<td>0.0001</td>
</tr>
<tr>
<td>TNF-α</td>
<td>r</td>
<td>0.112</td>
<td>0.788**</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>P-Value</td>
<td>0.47</td>
<td></td>
<td>0.0001</td>
</tr>
</tbody>
</table>

The Pearson correlation coefficient in a group of healthy subjects shows there was a strong correlation between TLR-9 and TNF-α with a p-value of 0.0001, while there is a moderate correlation between TLR-9 and IL-6 with a P-value of 0.006 and between TNF-α and IL-6 with P-value of 0.002 as shown in Table 4.

### Table 4. Correlation between TLR-9, TNF-α, and IL-6 in control.

<table>
<thead>
<tr>
<th>Parameters</th>
<th>Pearson Correlation</th>
<th>TLR-9</th>
<th>IL-6</th>
<th>TNF-α</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR-9</td>
<td>r</td>
<td>1</td>
<td>0.404**</td>
<td>0.613**</td>
</tr>
<tr>
<td></td>
<td>P-Value</td>
<td></td>
<td>0.006</td>
<td>0.0001</td>
</tr>
<tr>
<td>IL-6</td>
<td>r</td>
<td>0.404</td>
<td>1</td>
<td>0.445**</td>
</tr>
<tr>
<td></td>
<td>P-Value</td>
<td>0.006</td>
<td></td>
<td>0.002</td>
</tr>
<tr>
<td>TNF-α</td>
<td>r</td>
<td>0.613**</td>
<td>0.445**</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>P-Value</td>
<td>0.0001</td>
<td></td>
<td>0.002</td>
</tr>
</tbody>
</table>

The receiver operating characteristic (ROC) analysis can discriminate between two patient states, typically referred to as "diseased" and "non-diseased" so it may be used in clinical epidemiology to quantify how accurate medical diagnostic tests are. The ROC curve data of the immunological biomarker of the current study are shown in Table 5 and Fig. 1.

Sensitivity was correlated with patients as its significance is related to disease (true positive), while specificity was correlated with healthy people (true negative). In the current study, all immunological parameters (TLR-9, TNF-α, and IL-6) of AML patients were tested by the ROC curve to show any one of these three parameters is a good parameter to diagnose and monitor inflammation in AML patients. Results of the ROC curve recorded that all parameters show good sensitivity and specificity in monitoring inflammation and disease activity in AML patients and thus may act as a good immunological biomarker to target the disease. Table 5 shows the sensitivity and specificity in addition to the Area Under the ROC Curve (AUC) of TLR-9, TNF-α, and IL-6 which showed an excellent explanation with a P-value of 0.001. The best cut-off of parameters was 2.28, 135.43, and 30.72 for TLR-9, TNF-α, and IL-6, respectively.
Table 5. Sensitivity and specificity of immunological biomarkers

<table>
<thead>
<tr>
<th>Parameters</th>
<th>AUC</th>
<th>Explanation</th>
<th>P value</th>
<th>The best Cut off</th>
<th>Sensitivity %</th>
<th>Specificity %</th>
</tr>
</thead>
<tbody>
<tr>
<td>TLR-9</td>
<td>0.807</td>
<td>Excellent</td>
<td>0.001</td>
<td>2.28</td>
<td>98%</td>
<td>71%</td>
</tr>
<tr>
<td>TNF-α</td>
<td>0.950</td>
<td>Excellent</td>
<td>0.001</td>
<td>135.43</td>
<td>88%</td>
<td>91%</td>
</tr>
<tr>
<td>IL-6</td>
<td>0.937</td>
<td>Excellent</td>
<td>0.001</td>
<td>30.72</td>
<td>89%</td>
<td>95%</td>
</tr>
</tbody>
</table>

A- ROC curve of TLR-9, B- ROC curve of TNF-α, and C- ROC curve of IL-6.

Although there were several previous studies in Iraq and neighboring countries about measuring the level of IL-6 and TNF-α in the serum of AML patients, the current study was the first study to link these cytokines with the level of TLR-9 in AML patients including the study by Kadhum et al., (2022) 11. The WBC level of the current study was without significant differences for both patients and healthy subjects, respectively, although previous studies showed a high level of WBC in patients. The level of hemoglobin for this study was significantly different between patients and healthy control, where anemia was noticed in patients of all types without significant differences. The current study agrees with the study of Al-Husseiny where anemia was present for all patients without significant difference19. The result also corresponds to the study by Ahmed et al., which clarified significant differences between AML patients and controls (P=<0.001) in CBC parameters like hemoglobin and platelets while the result disagreed with the level of WBC20. The results of the current study showed that there were no significant differences between patients and control group in the number of white blood cells, despite the normal significant increase in white blood cells in patients with leukemia. However, the reason for this result may be due to the diversity of the group of patients, as some of them were in the initial stages of chemotherapy with a noticeable increase in white blood cells while the secondary AML and relapsed groups had a very low number of white blood cells, which led to a balance in the result that led to a value close to the normal value of WBC.

The current study showed elevated levels of IL-6 in AML patients compared with healthy controls, this result corresponds with previous studies like the Iraqi study by Kadhum et al, which clarified a
greater value of IL-6 for AML patients than controls. Another study showed that serum levels of IL-6 can be used as predictive serum markers at diagnosis of AML. The pathogenesis of various hematological malignancies, including AML, has been linked to cytokine dysregulation and plasma cytokine levels have been linked to disease progression and survival. Our findings are consistent with the previous study conducted by Sanchez-Correa et al., which found an increase in IL-6 in AML patients.

Cytokines are dissolved molecules produced either by the body or because of damage to the tissue or a particular disease such as cancer. AML cytokines were produced either by immune cells or blasts but until this moment, not known whether they have a role in increasing the severity of pathogenicity. AML has been linked to changes in cytokine levels, which have been linked to autoimmune illnesses, allergies, and cancer. Tumor formation is linked to an inflammatory environment, and tumors appear to be dynamic, interacting systems.

The levels of TNF-α showed a significant increase in the patients compared to the healthy controls, indicating a proinflammatory cytokine was correlated with an increased risk of AML. This result agrees with previous studies a study by Tsimberidou et al., which confirmed that elevated serum level of TNF-α was an adverse predictive factor for survival and Effective Free Persistence in high-risk MDS or patients with untreated AML. Wang et al, clarified that the level of plasma TNF-α significantly increased in newly diagnosed AML patients compared with healthy controls which correspond to the current result.

Tissue damage or cancer may cause a change in the activity and expression of TLR-9 and therefore possibly be used as a biomarker to treat cancer. This result showed an elevated level of TLR-9 in AML patients compared to controls with mean± SE was (2.728±0.04), (2.219±0.06) respectively. This result agrees with a previous study that showed TLR9 blood levels were greater in patients than in the control group and had a statistical significance difference (P-value 0.05), according to the study conducted on Pediatric Acute Lymphoblastic leukemia. TLR9 also was discovered to be greater in breast cancer patients in comparison to healthy controls that agrees with our study.

According to the current study, there was a significant positive correlation between IL-6 and TNF-α in patients indicating that the secretion of these cytokines was high in patients, but only a weak positive correlation between TLR-9 and TNF-α and a medium positive correlation between TLR-9 and IL-6 in healthy people this may indicate the complex environment of inflammation. The result of the current study indicated the stimulation of the immune response due to the secretion of these cytokines. The up-regulation of the inflammatory cytokine IL-6 gene expression in the tissue, the up-regulation of IL-6 in the blood, and the concentration of IL-6 in the serum can all be used as indicators of the likelihood of colorectal cancer (CRC) recurrence in humans with colorectal cancer and higher levels of TLR expression have been linked to these outcomes. The production of TNF-α may be controlled by TLR-7 and TLR-9 overexpression in serum, which may contribute to the progression and development of urinary bladder cancer. When compared to controls, patients' levels of TLR-9 and TNF-α expression were considerably greater. The elevated TLR-9 level in AML patients in the current study may be affected by the IL-6 and TNF-α serum levels due to stimulating signaling pathway dependent on TLR-9 classical pathway thus activate a cascade of signals using specific transcription factors such as nuclear factor kappa B cell (NF-κB).

TLR-9 expression in leukemic cells of AML patients with a very low percentage compared to healthy control according to previous a previous study in a neighboring country (Egypt) that contradicted the result of the current study. The expression of TLR-9 was negatively related to the clinical outcome. Cytokines affect the growth of all types of cells in the blood and body cells, which in turn help stimulate the immune response and inflammation. The production of cytokines is an indication of the presence of the immune response, so the positive association of some of these cytokines is evidence of their secretion in
abundance by immune cells, while the negative association may be due to the consumption of cytokine or its association in certain sites of tissues, and this is evidence that this link is the result of a deep inflammatory environment \(^3\).

**Conclusion**

There was a strong positive correlation between IL-6 and TNF-\(\alpha\) in AML patients, while there was a strong positive correlation between TNF-\(\alpha\) and TLR-9, and a medium positive correlation between TLR-9 and IL-6 in healthy control. According to the current study, the high serum levels of immunological parameters including TLR-9, IL-6, and TNF-\(\alpha\) in AML patients was an important indicator of an increase in the severity of disease and may affect patient’s response to chemotherapy that leads to a risk of poor outcome. An elevated level of these cytokines may be used as biomarkers to target AML cells as part of immunotherapy, due to these markers had a high sensitivity and specificity level, which was confirmed by the ROC Curve test.

**Acknowledgment**

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**Authors’ Declaration**

- Conflicts of Interest: None.
- We hereby confirm that all the Figures and Tables in the manuscript are ours. Furthermore, any Figures and images, that are not ours, have been included with the necessary permission for re-publication, which is attached to the manuscript.
- Authors sign on ethical consideration’s approval.
- Ethical Clearance: The project was approved by the local ethical committee in Medical City no.38035 at 26/10/2021

**Authors’ Contribution Statement**

M. Q. M. did the Sample collection, practical part, writing, and Publishing of the manuscript. A. H. A made the conception and design of the manuscript. A. A. A performed the data analysis, statistics, and proofreading.

**References**


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