RESEARCH ARTICLE

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# Investigation Role of Toll-like Receptor-9 Gene and *miR-155* Expression Levels in Acute Myeloid Leukemia via Quantitative Real-Time PCR

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## Abstract

**Objective:** This study aims to investigate the mRNA expression levels of Toll-like receptor 9 and microRNA-155 in Iraqi patients diagnosed with acute myeloid leukemia (AML) within a case-control study framework. Additionally, the study will assess relevant hematological parameters. **Methods:** This study enrolled 40 Iraqi patients diagnosed with AML who were undergoing chemotherapy and experiencing relapse, and 40 healthy individuals as a control group. Hematological parameters were measured using a complete blood count (CBC) device. RNA was extracted from samples, quantified, and assessed for purity. Subsequently, RNA was reverse-transcribed into complementary DNA (cDNA). The relative expression levels of TLR-9 and miR-155 genes were quantified using quantitative real-time polymerase chain reaction (qRT-PCR). **Result:** Hemoglobin, erythrocyte, hematocrit, and platelet levels exhibited significant differences between AML patients and healthy controls. In contrast, white blood cell and lymphocyte counts were not significantly different between the two groups. TLR-9 gene expression was comparable between healthy controls and AML patients, with fold change values of 1.000 and 1.49, respectively. However, miR-155 expression was significantly lower in AML patients compared to healthy controls, with fold change values of 0.608 and 1.000, respectively. **Conclusion:** To evade host immune surveillance, cancer cells may downregulate the expression of TLR-9 and miR-155. This dysregulation may contribute to the progression and development of AML. Furthermore, the downregulation of miR-155 and dysregulation of TLR-9 during oncogenesis may serve as potential prognostic markers for AML patients.

**Keywords:** Acute Myeloid Leukemia- Pattern Recognition Receptors- Toll-Like Receptor-9 gene- *miR-155*

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## Introduction

Leukemia is a type of blood cancer that arises from the bone marrow's dysfunction. It is characterized by the abnormal proliferation of white or red blood cells and can manifest as either an acute or chronic condition [1, 2]. Between 2005 and 2015, the global incidence of leukemia increased by 26% [3, 4]. According to the 2018 annual publication of the Iraqi Cancer Registry, leukemia was the third most common cancer among Iraqi males, with 1061 cases and an incidence rate of 7.79 per 100,000 population. Among females, leukemia ranked fourth, with 838 cases and an incidence rate of 4.68 per 100,000 population [5]. Acute Myelocytic Leukemia (AML) is a hematological malignancy characterized by the uncontrolled proliferation of immature myeloid cells, known as blast cells. Age and the specific biological characteristics of the disease are significant prognostic factors for AML. Left untreated, AML can be fatal [6]. Acute Myeloid Leukemia pathogenesis involves the abnormal growth and differentiation of myeloid stem cells. This can arise from underlying hematological disorders or as a consequence of prior treatments. Various genetic abnormalities, such as chromosomal translocations, deletions, or additions, play a crucial role in driving AML development. [7, 8].

Toll-like receptors (TLRs) are key components of the innate immune system, functioning as Pattern Recognition Receptors (PRRs). They play a crucial role in recognizing both non-pathogenic and pathogenic microorganisms, as well as in immune surveillance. TLRs can detect pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs), enabling them to initiate immune responses in response to external or internal threats [9]. The TLRs induce an immune response against tumors by stimulating antigen-presenting cells to produce pro-inflammatory cytokines and co-stimulatory molecules. This process can disrupt tumor

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tolerance, leading to the activation of anti-tumor immune responses [10]. While TLRs are known to be expressed or upregulated in various tumor cell lines and malignancies, their specific expression levels and functional significance in the genetics and progression of acute leukemia remain relatively unexplored [11]. Overexpression of TLRs has been linked to increased cell survival, tumor proliferation, and metastasis in several malignancies, including lung, colon, and breast cancers [12]. Toll-like receptor 9 (*TLR-9*) is a human protein encoded by the TLR9 gene located on chromosome 3p21.2. Previously identified as CD289, *TLR-9* belongs to the Toll-like receptor family. As an intracellular receptor expressed in immune cells such as dendritic cells, natural killer cells, macrophages, and other antigen-presenting cells, *TLR-9* recognizes viral and bacterial DNA. Upon activation, *TLR-9* triggers a signaling cascade that leads to the production of pro-inflammatory cytokines, ultimately contributing to tissue damage and cancer. Various factors, including tissue damage and cancer, can modulate the expression and activation of *TLR-9* [13, 14].

MicroRNAs (miRNAs) are short non-coding RNA molecules that regulate gene expression post-transcriptionally [15]. These 19-25 nucleotide RNAs play crucial roles in various cellular processes, including the maintenance of hematopoietic stem cells, apoptosis, gene methylation, cell cycle regulation, progenitor cell self-renewal, and myeloid differentiation [16, 17]. Recent research has elucidated the diverse physiological roles of miRNAs, ranging from cell cycle regulation to metabolism and aging. Dysregulation of miRNAs has been implicated in various human diseases, including cancer, diabetes, and neurodegenerative disorders, growing research has revealed the critical role of the miRNome, the entire set of miRNAs in a genome, in normal hematopoiesis. Specifically, miRNAs undergo specific alterations during the activation and differentiation of hematopoietic stem cells into various lineages [18].

The B cell integration cluster (BIC) long non-coding RNA transcript harbors the MIR155HG gene within its exon region. Located on chromosome 21q21.3, MIR155HG encodes *miR-155*, a miRNA with a crucial role in hematopoiesis, immunology, inflammation, and cancer. While *miR-155* has been implicated in the development of hematopoietic malignancies, its expression is essential for the maturation, differentiation, and normal growth of immune and hematopoietic cells. Moreover, *miR-155* plays a well-defined role in the homeostatic regulation of the immune system [19, 20]. Given the crucial role of TLRs in immune responses and their involvement in the pathogenesis of various cancers, this study aimed to investigate the expression levels of *TLR-9* in Iraqi patients diagnosed with AML. Additionally, the study sought to explore the role of *miR-155* expression levels in these patients.

## Materials and Methods

*Subjects*

Approximately 3 mL of whole blood was collected from forty Iraqi patients diagnosed with acute myeloid leukemia, encompassing both male and female individuals. Additionally, forty healthy individuals served as controls. The age range of the participants spanned from 15 to 83 years, including those undergoing chemotherapy (M2, M3, M4, and M5) and individuals experiencing relapse. Blood samples were collected between October 2021 and December 2021 from Al-Imamain Al-Kadhimin City Teaching Hospital and Baghdad Hospital in the Medical City, Baghdad. The study proposal is outlined in Figure 1.

*Haematological parameters*

A complete blood count (CBC) was performed to measure hematological parameters, including total red

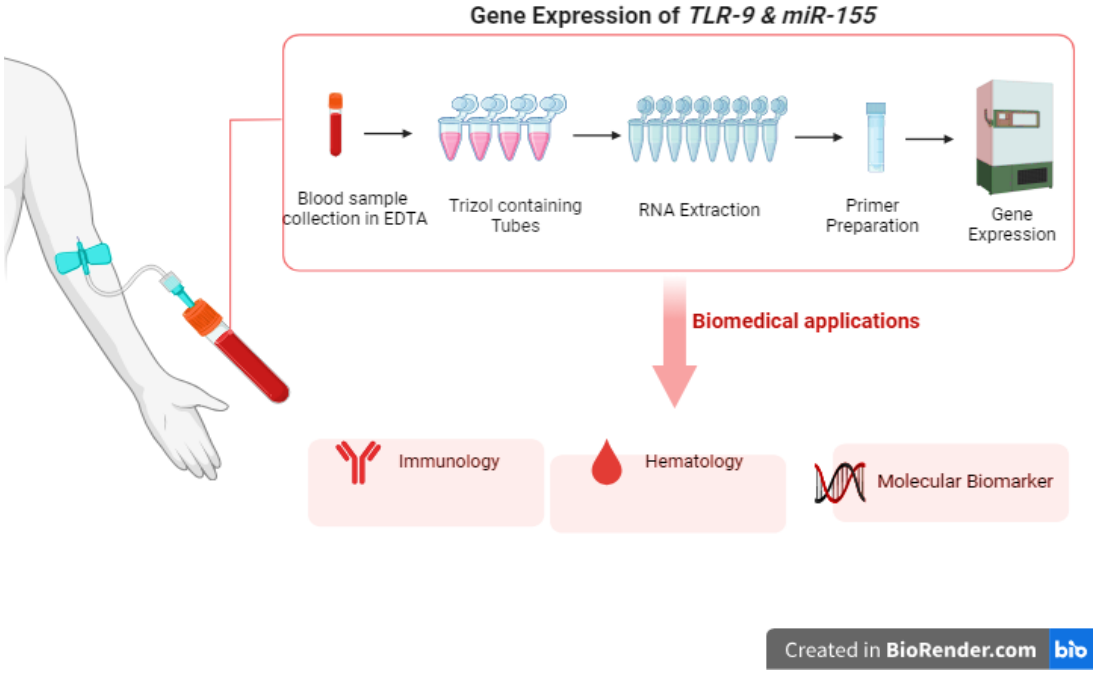


Figure 1. The Study Proposal of the Current Study

blood cell (RBC), white blood cell (WBC), lymphocyte, hemoglobin (HGB), hematocrit (HCT), and platelet counts. These measurements were obtained using an automated hematology analyzer manufactured by Mindray, Germany [21].

*Molecular Study*

*RNA Extraction, Concentration, and Purity Measurement*

Total RNA was extracted directly from whole blood samples using the Easy Pure® Blood RNA Kit (TransGen Biotech Company, China) with cat no.ER401, following the manufacturer's protocol. RNA concentration and purity were assessed using a NanoDrop spectrophotometer (Thermo Fisher Scientific, USA) to ensure the quality of the RNA samples for subsequent RT-qPCR analysis. The RNA extracted from the blood samples exhibited a concentration range of 60-140 ng/µl. Purity assessments, conducted at two wavelengths and expressed as an A260/A280 ratio, yielded values around 2.0, indicating high-quality RNA suitable for subsequent RT-qPCR analysis.

*Synthesis of cDNA*

The cDNA synthesis was performed using the One-Step gDNA Removal and cDNA Synthesis SuperMix (EasyScript®), following the manufacturer's protocol. The reaction mixture consisted of a reaction mix (10µl), random primers (1µl), anchored oligo dT (1µl), a genomic DNA remover (1µl), RNase-free water (3µl), E-mix reverse transcriptase (1µl), and a specified amount of total RNA (5µl),. Thermal cycling involves three stages: an initial denaturation step at 25°C for 10 minutes, a reverse transcription step at 42°C for 15 minutes, and a final enzyme inactivation step at 85°C for 5 seconds.

*Primers*

Primers specific to the *TLR-9*, *GAPDH*, *miR-155*, and *U6* genes were obtained from previous studies and synthesized by Alpha DNA Company (Canada). The primers were received in lyophilized form and reconstituted with nuclease-free water to a concentration of 1 pmol. The primer sequences and their respective references are provided in Table 1.

*Gene Expression*

Following the manufacturer's instructions, the reaction was carried out in a final volume of 20 µl. The reaction mixture consisted of 10 µl of SYBR Green Master Mix, 3 µl of cDNA, 1 µl of forward primer, 1 µl of reverse primer, and 5 µl of nuclease-free water. The thermal cycling conditions for gene expression are outlined in Table 2.

*Statistical analysis*

Mean and standard deviation values for both *TLR-9*, *GAPDH*, *miR-155*, and *U6* genes were calculated using Excel 2010. The relative expression levels of the *TLR-9* and *miR-155* genes were determined using the $2^{-\Delta\Delta Ct}$ method [25]. Hematological parameters were analyzed using Statistical Package for the Social Sciences (SPSS) version 26 [26].

Table 1. Primers Used in the Current Study

| Primer | Sequence (5′→3′ direction) | Product size(bp) | Tm (°C) | Ref. |
|---|---|---|---|---|
| TLR-9 | | | | |
| Forward | CAAAGGGCTGGCTGTTGTAG | 77 | 62 | -8 |
| Reverse | CCTCTACCACGAGCACTCAT | | 62 | |
| GAPDH (Glyceraldehyde 3-phosphate dehydrogenase( | | | | |
| Forward | CGGGTTCCTATAAATACGGACTG | 150 | 62 | -22 |
| Reverse | CCAATACGGGCCAAATCCGTTC | | 60 | |
| miR-155 | | | | |
| Forward | GCGGTTAATGCTAATCGTGATA | -- | 59 | -23 |
| Reverse | CGAGGAAGAAGACGGAAGAAT | | 59 | |
| U6 | | | | |
| Forward | AGAGAAGATTAGCATGGCCCCT | -- | 62 | -24 |
| Reverse | GCGAGCACAGAATTAATACGAC | | 58 | |

Table 2. Stages and Temperature of Teal-time qPCR for *GAPDH* and *TLR-9* Genes

| Stages | | Temperature °C | Time /Sec. | Cycle |
|---|---|---|---|---|
| First stage | Denaturation | 94 | 60 | 1 |
| Second stage | Denaturation | 94 | 5 | |
| | Annealing * | 58 | 15 | 40 |
| | Extension | 72 | 20 | |
| Third stage | Dissociation | 65-95 | | 1 |

*Annealing temperature for TLR-9, GAPDH, miR-155 an U6 were 58°C

## Results

*Demographic Characteristics*

Table 3 presents the gender distribution of the study participants. The control group comprised approximately equal numbers of males and females, with 40% being male and 60% female. In contrast, the patient group exhibited a higher proportion of males (67.5%) compared to females (32.5%). A statistically significant difference in gender distribution was observed between the two groups (p-value = 0.0084).

*Haematological Parameters*

Table 4 presents a comparison of white blood cell (WBC), lymphocyte, platelet (PLT), hemoglobin (HGB), red blood cell (RBC), and hematocrit (HCT) levels between patients and healthy controls. The results indicate no significant difference in WBC and lymphocyte counts between the two groups. However, significant differences were observed in hemoglobin, platelet, RBC, and hematocrit levels, with p-values of 0.0001 for each comparison.

Table 3. Distribution of Sample Study According to Gender in Different Groups

| Gender | Group | | P-value |
|---|---|---|---|
| | Patients No. (%) | Control No. (%) | |
| Male | 27 (67.50%) | 16 (40.00%) | 0.0084 ** |
| Female | 13 (32.50%) | 24 (60.00%) | 0.0084 ** |
| P-value | 0.0063 ** | 0.0097 ** | --- |

*TLR-9 Gene Expression*

Table 5 presents the mean CT values for all groups in the study. The mean CT value for AML patients was 19.612, while the mean CT value for the control group was 19.695. The housekeeping gene, *GAPDH*, exhibited mean CT values of 19.895 and 19.402 for AML patients and healthy controls, respectively. The ΔCt values for the patient and control groups were -0.283 and 0.293, respectively. The calculated fold change for *TLR-9* gene expression was 1.49, indicating no significant upregulation of gene expression in AML patients compared to the

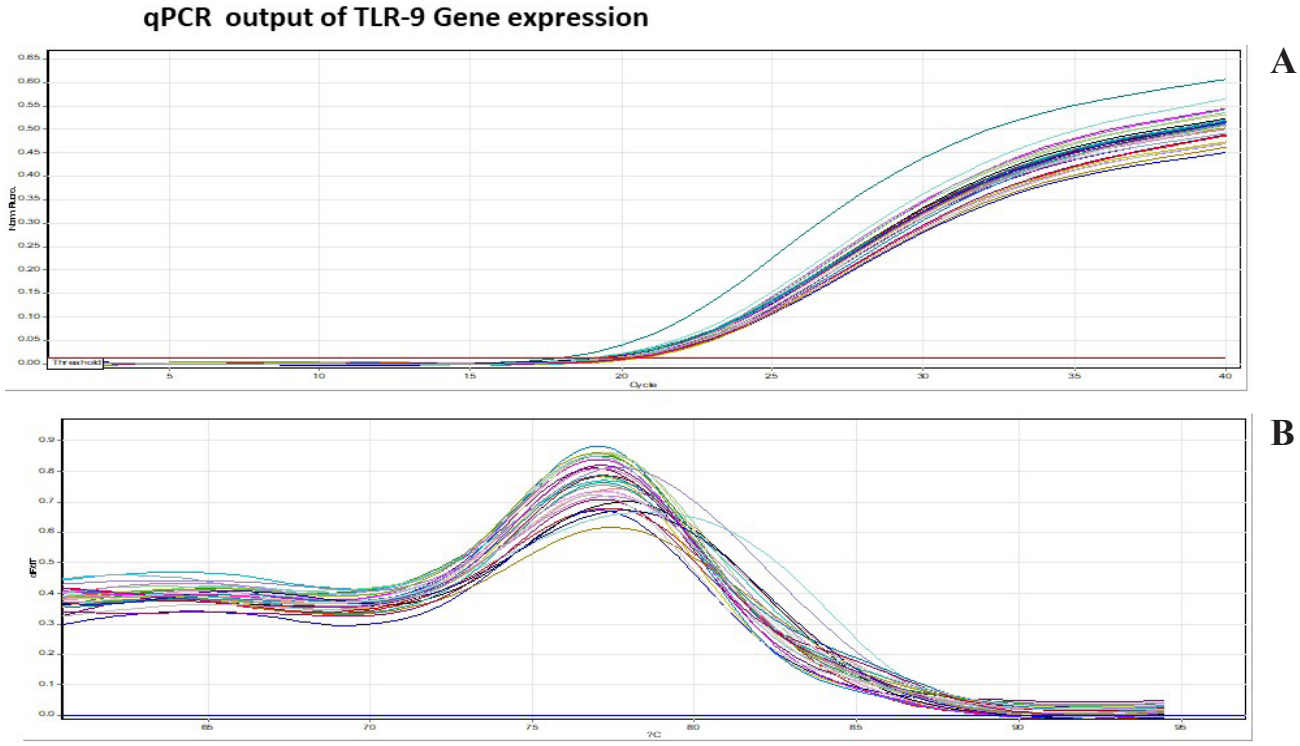


Figure 2. *TLR-9* Gene Expression Results (a) *TLR-9* amplification plots using Real-time qPCR. (b) The *TLR-9* dissociation gene curves by qPCR, The melting temperature ranged from 76°C to 77°C, and No primer dimer could be seen. The photograph was taken directly from the machine of Qiagen Rotorgene qPCR.

Table 4. Comparison between Patients and Control According to Study Parameters

| | Mean ± SE | | | | | |
|---|---|---|---|---|---|---|
| Group | WBC (x $10^9$ L) | Lympho. (x $10^9$ mL) | Hb (g/dl) | PLT (x $10^9$) | RBC (x $10^6$ mL) | HCT (%) |
| Patients | 10.55 ±2.41 | 4.34 ±1.88 | 8.34 ±0.33 | 69.78 ±12.20 | 2.89 ±0.12 | 25.07 ±1.00 |
| Control | 7.49 ±0.27 | 4.04 ±1.03 | 14.57 ±0.36 | 257.97 ±11.28 | 4.92 ±0.10 | 42.47 ±0.98 |
| T-test | 4.825 N.S | 4.097 N.S | 0.973 ** | 33.077 ** | 0.316 ** | 2.799 ** |
| P-value | 0.211 | 0.885 | 0.0001 | 0.0001 | 0.0001 | 0.0001 |

Table 5. Fold of *TLR-9* Gene Expression Depending on $2^{-\Delta Ct}$Method

| Study groups | Means Ct of *TRL-9* gene | Means Ct of *GAPDH* | ΔCt | $2^{-\Delta Ct}$ | Experimental group/ Control group | Gene expression fold |
|---|---|---|---|---|---|---|
| Patient | 19.612 | 19.895 | -0.283 | 1.217 | 1.217/0.816 | 1.49 |
| Control | 19.695 | 19.402 | 0.293 | 0.816 | 0.816/0.816 | 1 |

Table 6. Fold Expression of *TLR-9* Gene According to ΔΔct Calibrator Method

| Group | Means Ct of *TRL-9* gene | Means Ct of *GAPDH* | Δct | Δct calibrator | ΔΔct | $2^{-\Delta\Delta ct}$ | fold |
|---|---|---|---|---|---|---|---|
| Patients | 19.612 | 19.895 | -0.283 | 0.051 | -0.334 | 1.26 | 1.49 |
| control | 19.695 | 19.402 | 0.293 | 0.051 | 0.242 | 0.846 | 1 |

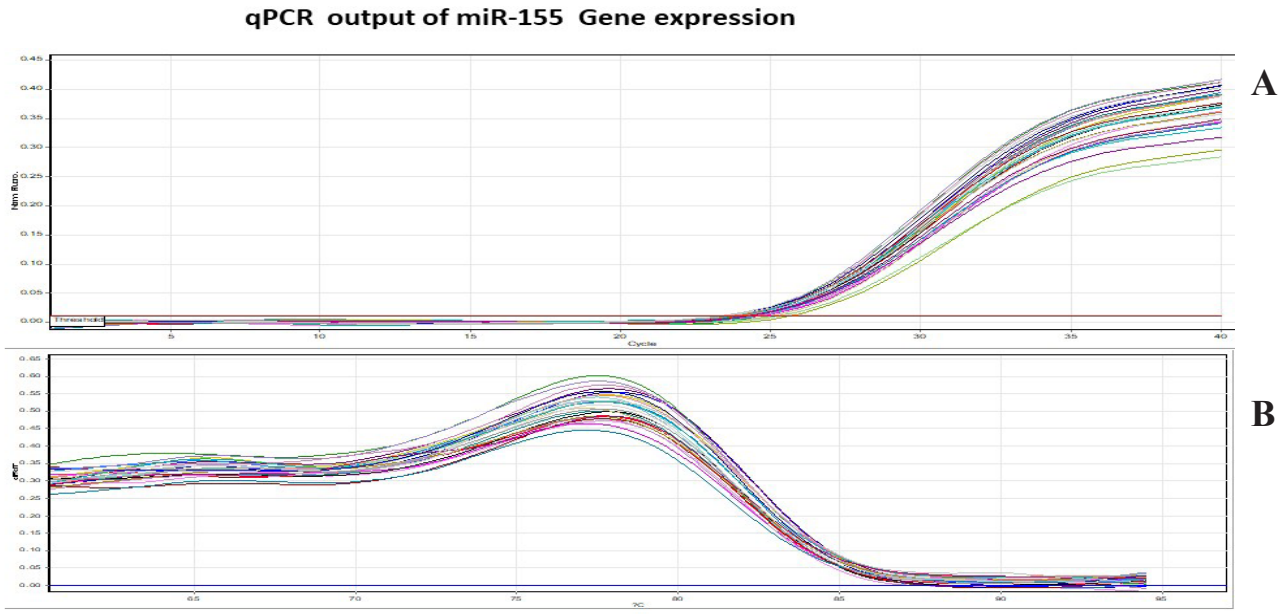


Figure 3. The *miR-155* Gene Expression Results. (a) *miR-155* gene amplification plots by qPCR. (b) The *miR-155* gene dissociation curves by qPCR, melting temperature ranged from 76°C to 77°C, No primer dimer could be seen.

control group in the Iraqi population. Table 6 presents the fold change in *TLR-9* gene expression, calculated using the ΔΔCt method. Both the patient and control groups exhibited the ΔΔct calibrator of 0.051. Figure 2 visually depicts the results of *TLR-9* gene expression.

*miR-155 Gene Expression*

Table 7 presents the mean CT values for *miR-155* in different groups. The mean CT value for AML patients was 24.503, while the mean CT value for the control group was 23.842. The housekeeping gene, *U6*, exhibited mean CT values of 17.323 and 17.38 for AML patients and healthy

Table 7. Fold of *miR-155* Gene Expression Depending on $2^{-\Delta Ct}$Method

| Groups | Means Ct of *miR-155* gene | Means Ct of U6 | ΔCt | $2^{-\Delta Ct}$ | experimental group/ Control group | The fold of gene expression |
|---|---|---|---|---|---|---|
| Patient | 24.503 | 17.323 | 7.18 | 0.007 | 0.007/0.011 | 0.608 |
| Control | 23.842 | 17.38 | 6.462 | 0.011 | 0.011/0.011 | 1 |

Table 8. Fold of *miR-155* Gene Expression Depending on Δct Calibrator Method

| Group | Means Ct of *miRNA155* gene | Means Ct of U6 | Δct | Δct calibrator | ΔΔct | $2^{-\Delta\Delta ct}$ | fold |
|---|---|---|---|---|---|---|---|
| Patients | 24.503 | 17.323 | 7.18 | 6.949 | 0.231 | 0.852 | 0.608 |
| control | 23.842 | 17.38 | 6.462 | 6.949 | -0.487 | 1.402 | 1 |

controls, respectively. The ΔCt values for the patient and control groups were 7.180 and 6.462, respectively. The calculated fold change for *miR-155* gene expression was 0.608, indicating a significant downregulation of gene expression in AML patients compared to the control group in the Iraqi population.

Table 8 presents the fold change in *miR-155* gene expression, calculated using the ΔΔCt method. Both the patient and control groups exhibited a ΔΔct calibrator value of 6.949. Figure 3 visually depicts the results of *miR-155* gene expression.

## Discussion

Host immunological responses, including the promotion of leukocyte proliferation within the bone marrow and lymphocyte expansion in peripheral tissues, are indispensable for effective defense against pathogens [27]. The regulation of cell proliferation and apoptosis is crucial for preventing cancer, as uncontrolled cell proliferation can lead to carcinogenesis, influenced by numerous endogenous and exogenous factors that govern cell survival and proliferation [28, 29]. The current study found that men are more susceptible to the disease than women. This disparity may be attributed to higher smoking rates among men and the potential influence of mutations in the tumor suppressor gene TP53, which are more prevalent in men. These findings align with previous research by Alwan et al. [30] and Ries et al. [31], which also demonstrated a higher susceptibility to AML among men. While previous studies have indicated a slight gender disparity in the prevalence of AML, the current study's findings suggest a more pronounced difference, with a significantly higher proportion of male patients compared to female patients [32, 33].

Hemoglobin levels exhibited significant differences between the patient and control groups, with anemia observed across all patient subtypes without significant variations. In contrast, the control group demonstrated normal hemoglobin levels. These findings align with Al-Husseiny's 2008 study, which reported anemia in all patients without significant differences [34]. The current study's results further support Ahmed's 2019 study, which reported significant differences ($P < 0.001$) in hemoglobin and platelet levels between AML patients and controls. However, the current study's findings contradict Ahmed's 2019 study regarding WBC counts, as no significant differences were observed in the current study [35]. According to the current study results, which support Haider's finding of a mean RBC value of $2.78 \pm 0.82$ for patients, the mean and standard error of the RBC parameter was $2.89 \pm 0.12$ for patients and $4.92 \pm 0.10$ for healthy controls, respectively [36].

The current study evaluated *TLR-9* mRNA expression in AML patients and found no upregulation, aligning with the theory that cancer cells may downregulate *TLR-9* to evade immune surveillance. This downregulation could potentially serve as a target for novel therapeutic approaches. While TLRs are often upregulated in various cancers and tumor cell lines, such as *TLR-4* and *TLR-5* in gastric cancer and *TLR-4* and *TLR-9* in lung and breast cancer, the specific role of *TLR-9* in AML warrants further investigation [28). A study by Morsi et al. demonstrated that *TLR-9* and *TLR-7* may be expressed in acute leukemia cells (lymphoblastic and myeloid). However, only *TLR-7* exhibited a significant increase in expression compared to controls, suggesting that *TLR-7* may play a crucial role in the immune evasion of acute leukemias (AML and ALL). In contrast, the mRNA expression of *TLR-7* and *TLR-9* was significantly lower in chronic myeloid leukemia (CML) patients compared to normal controls [37].

Numerous studies have focused on the relationship between the TLR signaling pathway and its downstream target genes as potential biomarkers for colon and rectal cancer. However, the underlying mechanisms remain unclear due to the differential activation of the TLR signaling pathway in cancer tissues compared to healthy cells [38, 39]. Previous studies, such as that by González-Reyes et al. [40] have demonstrated decreased *TLR-9* expression levels in cancer. In a study of 133 prostate cancer patients, high TLR9 expression was associated with abnormal cytokine levels and impaired cell cycle-induced apoptosis in cancer cells [40]. Furthermore, a study by Elmaagacli et al. reported that *TLR-9* may influence the outcome of AML patients who undergo hematopoietic stem cell transplantation from HLA-identical siblings [41].

It is now widely recognized that miRNAs can significantly impact a broad spectrum of leukemic activities, including chemotherapy resistance in various AML subtypes, as well as processes such as proliferation, survival, differentiation, self-renewal, and epigenetic control. miRNA expression profiles may provide valuable prognostic information [42]. While leukemia-associated genomic modifications are rich in miRNA sequences, only about 100 of these are expressed above background levels, suggesting that a limited subset of miRNAs plays a role in AML [43]. This study represents the first investigation in Iraq to demonstrate downregulation of *miR-155* expression in AML patients compared to controls. This finding suggests that *miR-155* may contribute to the pathogenesis of AML by enabling cancer cells to evade the immune system. As such, *miR-155* could potentially serve as a biomarker for the diagnosis and treatment of the disease. These findings are consistent with previous research, such as that conducted by Wang et al., which also reported decreased *miR-155* expression in AML patients compared to healthy controls [44]. According to a study by Abdelhalim et al., the downregulation of miR-150 in AML patients is essential for leukemogenesis [45].

According to Tang et al., overexpression of miR-29c may enhance the sensitivity of both non-resistant and resistant leukemic cells to the drug decitabine. Furthermore, the downregulation of miR-29c may serve as an independent prognostic biomarker in AML patients [46]. The miRNAs are known to be dysregulated during oncogenesis. Overexpression of *miR-155* has been associated with several cancers, including hematological malignancies. This suggests that *miR-155* may play an oncogenic role [20]. Malignancies are characterized by aberrant miRNA expression patterns and functional abnormalities. These expression patterns are often associated with the type, stage, and other clinical factors

of the disease. Consequently, the discovery of miRNAs holds significant potential for the development of novel diagnostic biomarkers and therapeutic targets for cancer treatment [47].

In conclusion, exploring the intricate association between *TLR-9* and *miR-155* offers a promising avenue for elucidating the molecular mechanisms underlying AML development. This knowledge could pave the way for the development of innovative therapeutic strategies and refined diagnostic approaches. The *TLR-9* gene expression is implicated in the inflammatory process, as it induces dysregulated cytokine and miRNA levels. Notably, *TLR-9* expression may constitute a component of the immune evasion strategy employed by malignant cells, playing a critical role in leukemogenesis and disease progression. While *miR-155* is well-recognized for its tumor-suppressive functions, it also exerts regulatory influence over a wide range of mRNA targets. This multifaceted regulation is essential for safeguarding against cellular transformation. Importantly, the downregulation of *miR-155* is a key event in the pathogenesis of leukemia. The expression pattern of *miR-155* is correlated with clinical disease variables and exhibits aberrant expression and functional abnormalities in AML. Consequently, *miR-155* emerges as a potential therapeutic target for the eradication of leukemic progenitors in conjunction with chemotherapy.

## Author Contribution Statement

All researchers contributed equally to all stages of the research, from study design to the final manuscript.

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*Ethical Approval*

This study was ethically approved by the Ministry of Health in Iraq (approval number 38035, issued on October 26, 2021) and by the Scientific Committee of Mustansiriyah University.

*Conflicts of Interest*

The authors declare no competing financial interests or personal relationships that could have influenced the research reported in this paper.

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