ISSN: 0067-2904

The Correlation Study between *TP53* Gene Expression and Acute Myeloid Leukemia in Iraq

Hadeel Hameed Dawood*, Rana Kadhim Mohammed
Department of Biotechnology, College of Science, University of Baghdad, Baghdad, Iraq

Received: 30/11/2022 Accepted: 18/1/2023 Published: 30/11/2023

Abstract

Acute myeloid leukemia represents the most prevalent type of acute leukemia in adults .Mutations in the tumor protein (TP53) gene have been found in more than half of all human cancers. This study was done to investigate the relationship between *TP53* gene expression and the appearance and progression of acute myeloid leukemia in Iraq. This study included 100 subjects, divided into 60 patients suffering from pre-diagnostic acute myeloid leukemia and 40 healthy individuals. The difference in *TP53* gene expression between acute myeloid leukemia patients and healthy individuals has been investigated, and the gene expression of *TP53* has been measured after extraction of total RNA at concentrations (15–83 ng/µl) and purity (1.76-2). Gene expression has involved calculating the double ΔCt value to assess *TP53* expression in the presence of the *GAPDH* gene as a reference gene. Results showed that gene expression folding ($2^{-\Delta\Delta Ct}$) reflects significant differences in *TP53* gene expression. There was overexpression (up-regulation) of the *TP53* gene in acute myeloid leukemia patients (mean ± SD: 2.29±2.12) compared with controls (1 ± 0.43), with a p=0.04.

Keywords: Acute myeloid leukemia, Gene expression, qPCR, Tumor protein 53(*TP53*).

دراسة العلاقة بين التعبير الجيني *TP53* وسرطان الدم النخاعي الحاد في العراق

هديل حميد داود* ، رنا كاظم محمد

قسم التقنيات الإحيائية، كلية العلوم ،جامعة بغداد ،بغداد ،العراق.

الخلاصة:

يمثل سرطان الدم النخاعي الحاد أكثر أنواع سرطان الدم الحاد انتشارًا بين البالغين. تم العثور على طفرات في الجين المثبط للورم في أكثر من نصف الامراض السرطانية في الانسان. هدفت هذه الدراسة الى الكشف عن العلاقة بين التعبير الجيني لجين بروتين الورم 53 وظهور وتطور سرطان الدم النخاعي الحاد في العراق. تضمنت هذه الدراسة 100 شخص مقسمة ال 60 مريض يعانون من سرطان الدم النخاعي الحاد في مرحلة التشخيص الاولي و 40 شخصا سليما. تمت دراسة الاختلاف في التعبير الجيني لجين بروتين الورم 53 بين مرضى سرطان الدم النخاعي الحاد والأشخاص الاصحاء وتم قياس التعبير الجيني لجين بروتين الورم 53 بعد استخلاص الحمض النووي الريبوزي الكلي بتركيز (15−83ng/µl) ونقاوة (1.76−2). يتضمن التعبير الجيني حساب قيمة ΔCt لتقييم تعبير بروتين الورم 53 بوجود جين *GAPDH*

*Email: hadeelhameed317@gmail.com

كجين مرجعي. اظهرت نتائج التعبير الجيني وجود فروق معنوية في تعبير جين بروتين الورم حيث وجد ان مرضى سرطان الدم النخاعي الحاد لديهم مستوى تعبير اعلى (mean± SD: 2.29±2.12) مقارنة بالأشخاص الأصحاء (mean± SD:1 ±0.43) مع p=0.04

1. Introduction

Acute myeloid leukemia (AML) is the most common type of leukemia in adults. It is a disorder in which undifferentiated myeloid precursors (blasts) grow quickly in the bone marrow and peripheral blood, causing the bone marrow to fail and erythropoiesis to work less well [1, 2]. Hematopoietic stem cell genetic and epigenetic alterations have been found to be crucial in AML pathogenesis [3]. Several studies show that 70% of the repeated mutations in AML patients are caused by gene expression regulators like splicing machinery parts, transcription factors, and epigenetic proteins [4]. The tumor suppressor gene (*TP53*) is present on the short arm of chromosome 17 (17p13.1) and encodes the protein p53. In response to cellular stress like DNA damage, there are increases in the levels of transcription genes that are responsible for DNA damage repair, cell cycle arrest, and apoptosis. As a result, defects in the p53 protein's function are expected by mutations of this "guardian of the genome," allowing cells to be underlying for programmed cell death (apoptosis) to avoid it and promote the development of cancerous diseases [5, 6]. Mutations in the *TP53* gene are found in about half of human malignancies and about 20% of newly diagnosed AML [7]. The progressive excess of altered patterns of gene expression as a result of genetic aberrations and epigenetic programs is a critical issue in the molecular understanding of tumorigenesis. Several elements are known to be involved in gene expression regulatory mechanisms. Some have been investigated for decades, and their mechanisms are well understood. Others are still being worked out. As it turns out, many things affect gene regulation in malignancies. These include direct changes in the DNA sequence (inversions, deletions, and insertions; gene fusions; copy number variation; translocations; single nucleotide variants, etc.) that affect the sequences of genes, regulatory sequences, or transcription factor genes, as well as changes in post-translational and epigenetic factors [8, 9]. The accumulation of knowledge about epigenetics and genetics in leukemia allows for the identification of new potential biomarkers and the investigation of new targeted therapies [10]. The goal of this research is to determine the *TP53* gene's expression in the blood samples collected from pre-diagnosed acute myeloid leukemia patients and apparently healthy controls.

2. Materials and Methods

2.1 Blood samples from leukemia patients: One hundred human blood samples, including forty from healthy control adults and sixty from adults diagnosed with acute myeloid leukemia, were taken from Baghdad Teaching Hospital at Baghdad Medical City (from March 2021 to September 2021). Whole blood samples were collected in EDTA tubes, and 200 µl of each blood sample was mixed with 600 µl of Genezol in an Eppendorf tube.

2.2 Extraction of RNA and synthesis of cDNA: Total RNA extraction (mRNA) was performed from samples using the GENEzol ^{TM} Reagent (Geneaid, Korea) kit by adopting the manufacturer's protocol. The absorbance of extracted RNA at 260/280 nm has been measured to determine its purity and concentration by using the NAS-99 spectrophotometer. Complementary DNA (cDNA) has been synthesized from 5 µl of RNA by using the *Transcript* One-Step gDNA Removal and cDNA Synthesis SuperMix kit.

2.3 Primers selection and preparation: Two primers have been used for estimation of *TP53* expression: a specific primer for the *TP53* gene and another primer specific for the housekeeping gene, referred to as glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), a

glycolytic enzyme, and the transferring receptor, which is used as a reference gene (internal control) for calculating the ΔCt value [11]. These two primers were obtained in lyophilized form from the Bioneer Company (Korea). Table 1 shows the sequences of these primers. Stock solutions with 100 pmoles/µl of each primer have been prepared from the lyophilized form of primers after dissolving in 150 µl of nuclease-free water based on the manufacturer's instructions and being preserved at -23 °C. The 10 pmoles/µl of work solution has been made by diluting 10 µL of primer stock solution in 90 µL of nuclease-free water and preserving it at -23 °C until use.

Table 1: Primers used in the study

Primer F: Forward	Sequence (5′→3′) direction
***TP53*-Tumor protein**	
F	AGGCTGTGTTCAAGTGGTTCTGA
R	ACAAAAATTAGCGGGGCGTGG
***GAPDH*- Glyceraldehyde 3-phosphate dehydrogenase 160 bp58C**	
F	GAAATCCCATCACCATCTTCCAGG
R	GAGCCCCAGCCTTCTCCATG

2.4 Relative quantitative PCR conditions and analysis: PCR reactions have been done using the TransStart Top Green qPCR Super Mix Kit to detect the synthesis of dsDNA. Reactions have been done in 20 µl volumes, including 1 µl of every primer, 3 µl of cDNA, 5 µl of nuclease-free water, and 10 µl of Green Master Mix Reagent. The PCR program conditions in this study include a step of denaturation at 94 °C for 30 seconds, then 40 cycles of denaturation at 94 °C for 5 seconds, annealing at 58 °C for 15 seconds, extension at 72 °C for 20 seconds, and finally the melting curve at 65–95 ºC.

2.5 Calculation of *TP53* gene expression

Expression levels have been normalized against housekeeping genes. The *GAPDH* and levels of gene expression have been calculated according to the real-time quantification method [12], in which the fold change is used to report the results. The $2^{-\Delta\Delta Ct}$ method has been utilized for assessing the level of transcription between various samples. The target gene's CT value has been standardized to that of the internal control gene. The differences in values of cycle threshold (Ct) between *GAPDH* (the housekeeping gene) and *TP53* (the target gene) have been assessed. The following equations summarize the optimal calculations to find the folding for any gene and compare it to the groups it has been applied to.

$$\text{Folding}=2^{-\Delta\Delta Ct}$$

$$\Delta Ct\ (\text{Test}) = Ct_{\text{target gene}} - CT_{\text{internal control.}}$$

$$\Delta\Delta Ct = \Delta Ct_{\text{target gene}} - \Delta Ct_{\text{internal control}}$$

2.6 Statistical analysis: One-way ANOVA and the T-test were used to significantly compare means and parametric variables. The results have been reported as the mean ± standard deviation. The differences have been deemed significant if p values <0.05. Statistical analysis has been done using IBM SPSS 66 Statistics [13, 14].

3. Results and Discussion

3.1 Quantification of *GAPDH* expression by qPCR: The *GAPDH* gene is the housekeeping gene that has been used in this study. The results of the Ct value for the *GAPDH* gene between pre-diagnostic AML and control groups ranged from 23 to 25, with mean ±SD Ct values of (24.23 ± 0.74) and (24.29 ± 0.56) in the pre-diagnostic AML and healthy groups, respectively, and there were no significant differences in the Ct value means of GAPDH between these two groups (p = 0.67), as mentioned in Table 2. The *GAPDH* gene dissociation curves and amplification plots are shown in Figure 1, A, and B.

Table 2: Comparison between study groups regarding *GAPDH* fold expression levels

Group	Ct value of *GAPDH* (Mean ± SD)	Fold expression
Group 1: AML-Patients	24.23 ± 0.74	2. 29 ± 2.12
Group 2: healthy	24.29 ± 0.56	1 ± 0.43
p-value	0.67 NS	0.04*

The use of housekeeping genes in investigations of molecular biology depends on the assumption that their expression levels remain constant from one cell to another, from one sample to another, from therapy to therapy, and from one patient to another. The *GAPDH* gene is one of the most widely used housekeeping genes in comparisons of gene expression. The expression of 1.718 genes in 72 types of normal human tissue was investigated using qRT-PCR with *GAPDH* as a reference gene. When used in clinical studies, they found that using the *GAPDH* gene as a normalization approach in qRT-PCR is a very dependable method [15].

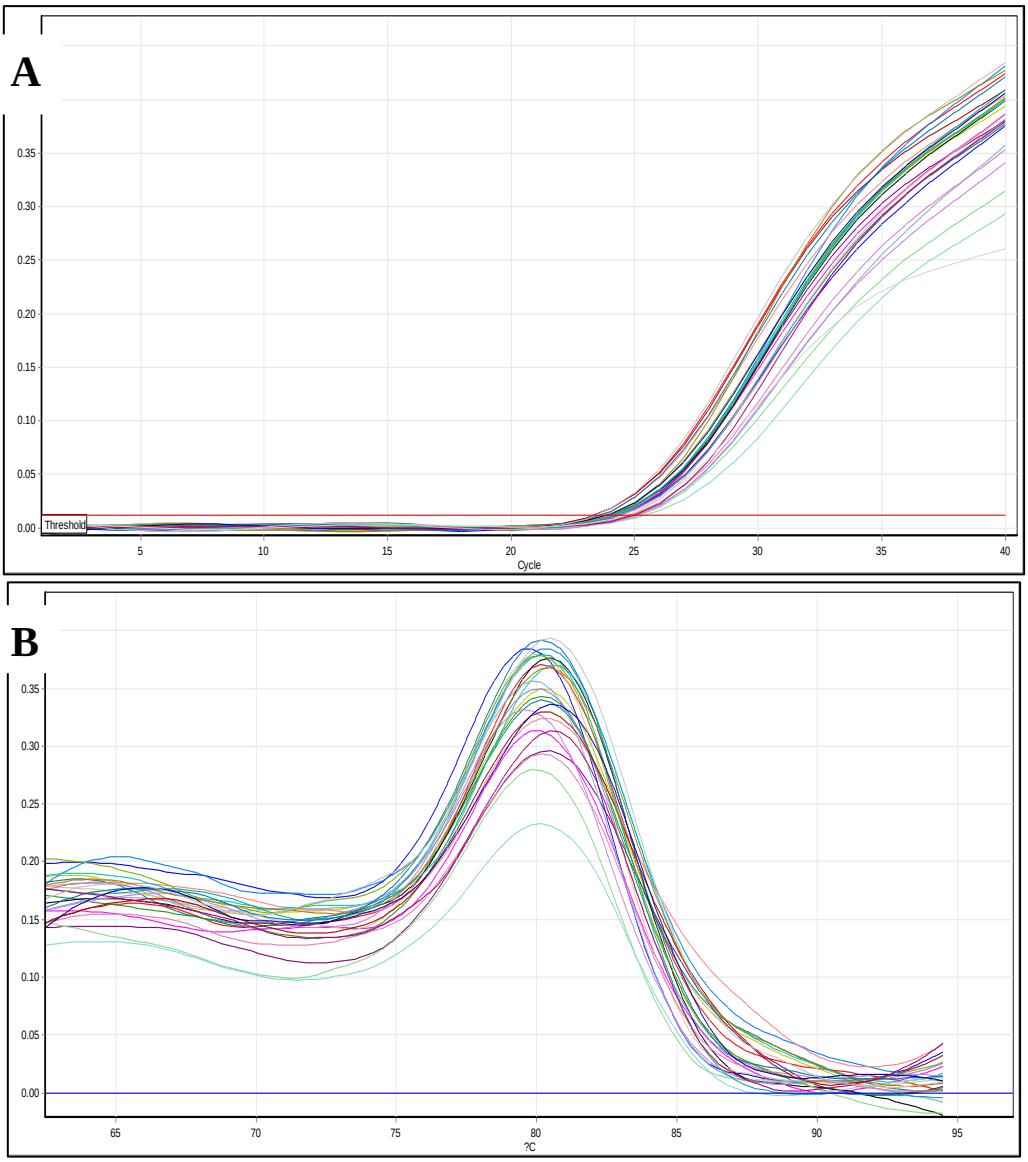

Figure 1: A. GAPDH gene amplification was plotted using qPCR samples from all research groups.

CT values ranged from 23–25; B. GAPDH gene dissociation curve using qPCR samples that covered all research groups The melting temperature varied from 78 to 83 °C. These images were captured using the Qiagen Rotor Gene Q qPCR apparatus.

3.2 Quantification of *TP53* expression by qPCR: Amplification of the target gene has been achieved by real-time PCR. The results showed the mean ± SD Ct values of *TP53* cDNA amplification in pre-diagnostic AML patients and control groups (29.60± 2.81 and 30.86± 2.38, respectively), as mentioned in Table 3. The results of the mean Ct differed significantly between these two groups. The mean Ct values in the healthy group were higher than in the patient group. This result reflects the original mRNAs present in the samples. Furthermore, the patient group is associated with an increased copy number of mRNA, suggesting its higher expression in the pre-diagnosis AML patient group compared to the healthy group, and increased expression of the *TP53* gene in all patient groups, suggesting that *TP53* can be employed as a diagnostic marker for AML.

Table 3: Comparison of *TP53* gene in Ct, ΔCt values (Mean ± SD) between investigation groups

Study Groups	Mean ± SD of		
	TP53 Ct value	*GAPDH* Ct value	*TP53* ΔCt value
Group 1: Patients	29.60± 2.81	24.23± 0.74	5.50± 2.26
Group 2: Healthy	30.86± 2.38	24.29 ± 0.56	6.28± 2.31
p-value	0.02*	0.67 NS	0.02*

For every sample, each quantitative PCR reaction has been performed twice. Acute myeloid leukemia patients' and controls' samples were run alongside non-primer and non-template controls. This is necessary to do the statistical computation for both groups and to provide a calibrator. Each run has plots, including amplification plots and dissociation curves. Figure 2A and 2B show the amplification plots and dissociation curves for the *TP53* gene.

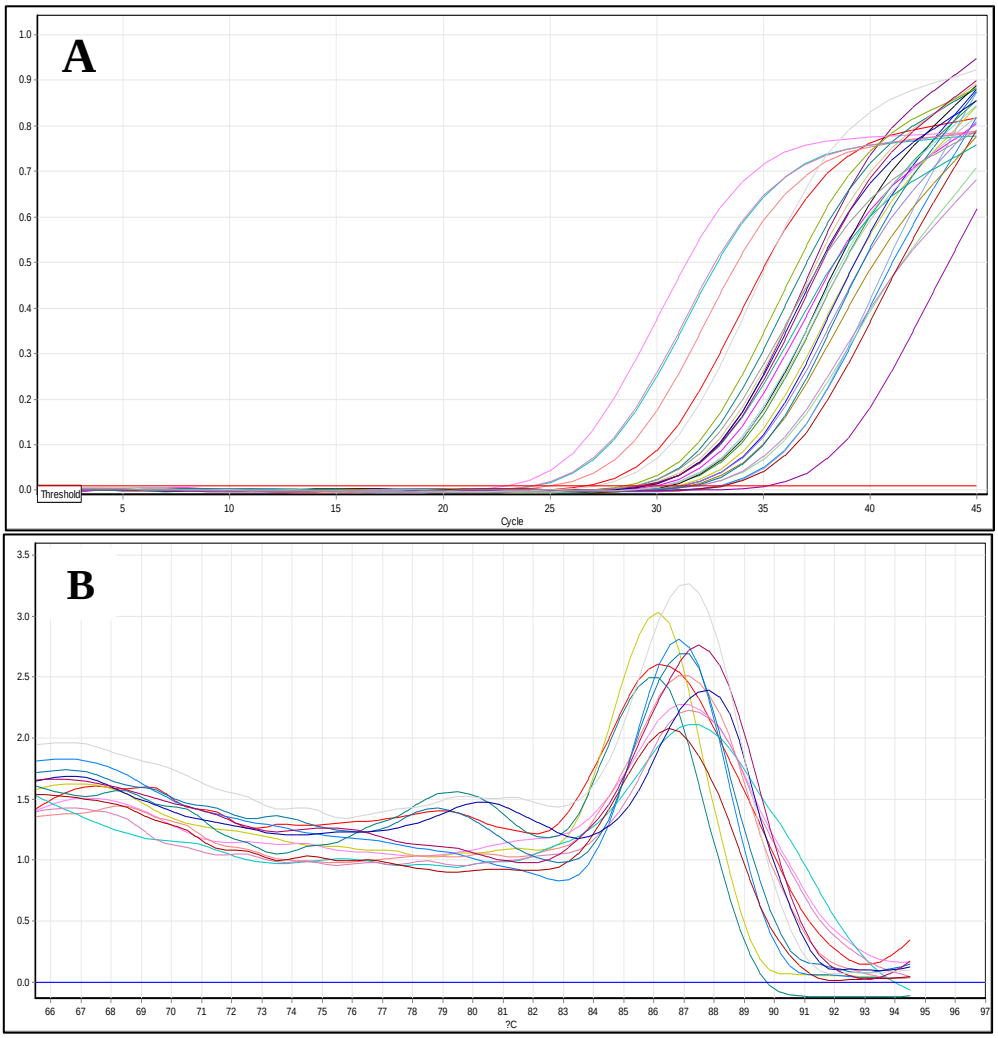

Figure 2: A. *TP53* gene amplification has been plotted by using qPCR samples that covered all research groups. The Ct values were different between 23 and 35. **B.** *TP53* gene dissociation curves using qPCR samples that covered all research groups. The melting temperature is different from 86–87 °C. These images were captured by the Qiagen Rotor Gene Q qPCR.

3.3 The *TP53* gene's Ct (cycle threshold) values normalization: A quantitative real-time PCR test has been used in this study to evaluate *TP53* gene expression and compare it between acute myeloid leukemia patients and healthy control people. The relative quantification equation has been used to determine gene expression fold changes [16]. This is based on normalizing the Ct values of the *TP53* cDNA to the *GAPDH* (it is conceded ΔCt). The mean of ΔCT (Ct values normalized) for every group in this study is mentioned in Table 4. The ΔCt means were (5.50± 2.26) in the pre-diagnostic AML patient group and (6.28 ± 2.31) in the healthy group, with significantly different (p = 0.02).

The $2^{-\Delta\Delta Ct}$ were used by each study group to assess the *TP53* gene expression. The results showed that the $2^{-\Delta\Delta Ct}$ was (2.29 ± 2.12) in the pre-diagnostic AML patient group, while the mean value of the $2^{-\Delta\Delta Ct}$ was (1± 0.43) in the healthy group. It was found that the mean of $2^{-\Delta\Delta Ct}$ differed significantly between these two groups (p = 0.04), as mentioned in Table 4. That means the $2^{-\Delta\Delta Ct}$ mean of AML patients yields the highest fold of *TP53* gene expression as compared with the healthy group. The *TP53* gene is overexpressed in AML, as in many other solid tumors. This result largely agrees with previous work showing that p53 is overexpressed in myeloid neoplasms [17].

In normal cells, the tumor suppressor protein *TP53* has a very limited half-life and is present in small amounts inside cells. It's stabilized in the nucleus in response to different kinds of stress and operates primarily as a transcription factor to activate genes involved in senescence, apoptosis, and growth arrest and also improve DNA repair based on the kind and strength of the signal. Changes in the *TP53* gene are the most prevalent genetic alterations identified in human malignancies. The mutated *TP53* gene has a longer half-life than the normal p53 protein. The mutation of the *TP53* gene often results in overexpression of the p53 protein [18]. Many tumors caused by mutations in *TP53* express high levels of mutant p53 protein and have a loss of the other *TP53* allele. In fact, high levels of mutant *p53* protein expression can be employed as a diagnostic marker for cancers caused by *TP53* gene mutations [19]. Three different things can result in tumorigenesis when the mutant p53 protein is overexpressed: 1. loss of activity of wild-type p53; 2. dominant negative effects on the wild-type p53 protein early in transformation prior to loss of the wild-type *TP53* allele; 3. mixed tetramer formation including both mutant and wild p53 proteins; and 3. de novo gain of functions regulated by mutant p53 protein that interacts with other tumor suppressors (e.g., p63, p73) and transcription factors [20].

Table 4: The *TP53* expression fold based on $2^{-\Delta\Delta Ct}$ method

Study Groups	Mean ± SD of			
	TP53 Ct value	ΔCT	ΔΔCT	$2^{-\Delta\Delta Ct}$
Group 1: Patients	29.60 ± 2.81	5.50± 2.26	2.81± 2.62	2.29 ± 2.12
Group 2: Healthy	30.86± 2.38	6.28± 2.31	2.42± 2.31	1 ± 0.49
p-value	0.02*	0.02*	0.02*	0.04*

4. Conclusions

The gene expression of *TP53* was determined in this study with 40 apparently healthy individuals and 60 AML patients using *GAPDH* as a reference gene. The results showed that there was no significant correlation between Ct values of the *GAPDH* gene in AML patients and control groups with p = 0.67, but there was a significant correlation between Ct values of

the *TP53* gene in AML patients and control groups with $p = 0.04$; thus, from this study, it can be concluded that there is increased *TP53* gene expression in AML patient groups as compared to control groups.

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