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LTB4R Gene Expression in Chronic Myeloid Leukemia in Iraq

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Abstract

The current study was carried out to explore gene expression of the LTB4R gene with the development of chronic myeloid leukemia (CML) in Iraqi patients. The differences in the expression of this gene between patients and healthy controls were studied. The correlation of gender and age with CML patients compared with controls was included as well as the correlation of gene expression folding $2^{-\Delta\Delta Ct}$ of LTB4R with clinical parameters (WBC, RBC, haemoglobin, platelets, and BCR-ABL gene). Results revealed significant increases in the mean of gene expression level (ΔCt) of patient groups compared to the corresponding ΔCt means in the healthy control group, the gene expression folding ($2^{-\Delta\Delta Ct}$) of the *LTB4R* gene reflects significant differences in the expression showed the highest level in CML patients which reached to (9.12), no significant differences were exhibited according to age and gender between CML patients and control. The study revealed a non-significant positive correlation between LTB4R gene expression level with both the BCR-ABL gene and WBC. Our results concluded that the *LTB4R* gene expression level could act as a marker for the prognosis of CML.

Keywords: CML, LTB4R, RT-PCR.

التعبير الجيني لجين LTB4R في مرضى ابيضاض الدم النقياني المزمن في العراق

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الخلاصة

أجريت هذه الدراسة للتعرف على التعبير الجيني للجين *LTB4R* في تطور مرض ابيضاض الدم النقياني المزمن في العراق وتم دراسة الفروقات في التعبير الجيني لهذا الجين بين المرضى ومجموعة الاصحاء ودرست العلاقة بين عمر وجنس المرضى بحدوث ابيضاض الدم النقياني ومقارنته مع مجموعة السيطرة .أظهرت النتائج وجود زيادة معنوية في متوسط التعبير الجيني للمرضى بالمقارنة مع السيطرة ويظهر التعبير الجيني للوكترين *LTB4R* فروقات معنوية في متوسط التعبير الجيني للمرضى وبمستوى عالي وصل الى (9.12) مع وجود فروقات معنوية .واضافة لذلك لم تظهر فروقات معنوية في التعبير الجيني بين المرضى والاصحاء تبعا الى العمر والجنس مما يشير الى ان مرض ابيضاض الدم النقياني المزمن يمكن ان يصيب كافة الاعمار ويحدث في كلا الجنسين الذكور والاناث . أظهرت النتائج وجود ارتباط موجب غير معنوي بين مستوى التعبير الجيني للجين *LTB4R* مع الجين BCR–ABL ومع عدد خلايا الدم البيضاء ويمكن الاستنتاج ان مستوى جين *LTB4R* يعد مؤشرا للتنبؤ بابيضاض الدم النقياني المزمن.

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1. Introduction

CML is a form of malignancy and proliferative disorder of the blood originating from myeloid progenitor cells in the bone marrow, resulting in a chromosomal abnormality known as the Philadelphia chromosome t(9;22)(q34q11) [1] . The production of a hybrid BCR-ABL is the molecular result of this chromosomal translocation. An immature myeloid cell, which produces red blood cells, platelets, and most types of white blood cells except lymphocytes, undergoes genetic change [2] . Philadelphia chromosome leads to the formation of the fusion gene BCR/ABL and the transcription of the fusion protein BCR/ABL in transformed cells [3, 4] . The disease is typically triphasic with a chronic phase (CP), an accelerated phase (AP), and then a short-duration blast phase [5, 6] . Importantly CML in children is rare [8] . In Iraq, the age distribution in CML patients is ranging between 14-70 years with a median age of 37 years [7].

Leukotrienes (LT) are biologically active lipid mediators that play a role in inflammatory and cancerous diseases [9]. Certain, leukocytes, primarily of myeloid origins such as; neutrophil, basophil, eosinophil granulocytes, mast cells, monocytes, and macrophages produce Leukotrienes, These cells express the components of the enzyme cascade required for the LT synthesis continuously and contain significant amounts of esterified arachidonic acid [10] . Cell proliferation; survival, migration, and metastasis are aided by the expression of leukotriene B4 receptors 1 and 2. LTB4R is highly expressed in all types of blood cells, but especially in myeloid cells like neutrophils and monocytes. LTB4R is a G-protein-coupled receptor with a high affinity for LTB4. LTB4R gene is located on chromosome 14q11.2-12 and consists of three exons and two introns [11] Leuktrain B4 exerts its biological action through two receptors known as BLT1 and BLT2 [12] . LTB4R1 and LTB4R2 have complicated structures and are highly polymorphic [13] . Leukotriene A4 hydrolyze is expressed in cells lacking 5-LO like endothelial, erythrocytes, fibroblast, and T cells.

LTB4R/LTB4R2 expression could be used as a predictor of tumors sensitivity to leukotriene receptor inhibitors [14] . This study aims to investigate the gene expression of LTB4R and it is a correlation with other clinical parameters as a predictive biomarker for CML progression and development in a sample of Iraqi patients.

2. Materials and Methods

Duration of the study and Ethics improvement

The blood sample collection and the practical work of this study extended over the period of five months from October 2020 to February 2021. The College of Science, University of Baghdad Ethical Committee approved this study at reference number CSEC/1020/0036

Patients and Control

Fifty CML patients were included in this study with an age ranging between (4-75) years old divided according to gender into (19) male and (31) female, samples; were obtained from Mosul city (Iben Sina Hospital) and Erbil city (Nanakaly Hospital), and were diagnosed with CML based on the complete blood picture (CBC) along with bone marrow examination, and *BCR-ABL* gene test is done by PCR technique. In addition, fifty controls with an age ranging from 20 to 65 years old were included (27 male and 23 female).

Blood Collection

Blood samples (5 ml) were collected from the subject and directly were put in the TRI zol TM Reagent-containing tube for RT-qPCR analysis.

Total RNA Extraction with TRIzol: RNA was isolated from the sample according to the protocol of TRIzolTM Reagent as the following:

Sample lysis: For each tube, 0.5 mL of blood was added to 0.5 mL of TRIzol TM Reagent, and the lysate was homogenized by pipetting up and down several times.

Three phase separation: Each tube's lysate was treated with 0.2 mL chloroform before the tube cap was secured. To separate the lower organic phase, interphase, and colourless upper aqueous phase, all of the mixtures were incubated for 2–3 minutes before being centrifuged for 10 minutes at 12,000 rpm. A new tube was filled with the aqueous phase containing the RNA.

RNA precipitation: In the aqueous phase, 500µl of isopropanol was added and left for 10 minutes before centrifugation at 12,000 rpm for 10 minutes. Total RNA precipitated into a white gel-like pellet at the bottom of the tube. After that, the supernatant was discarded.

RNA washing: For each tube, 0.5mL of 70% ethanol was added and vortexed briefly then centrifuged for 5 minutes at 10000 rpm. Ethanol then aspirated and air-dried the pellet.

RNA solubility: pellets were incubated in a water bath or heat block set at 55–60°C for 10–15 minutes after rehydration in 100µl of Nuclease Free Water.

RNA purity and concentration measurements: The purity and concentration of the extracted lysates are measured using a spectrophotometer Nanodrop (Q5000 (UV-VIS)). The purity is calculated by calculating the optical density (OD) ratio at 260/280nm. while the concentration is measured in ng/ml (absorption wavelength of protein and DNA). The purity of RNA that is accepted is between 1.7 and 2.
By using the equation. Vn=Co*V0/Cn,

The concentration of RNA samples is normalized to the lowest concentration of the sample. Where, Vn is the volume of the current sample that will be diluted by DW to generate a total of 100 µL, VO is the normalized volume (equal to 100µL and Co is the lowest concentration.

Primer design: The NCBI Gen Bank database was used to obtain the cDNA sequences of the *LTB4R* gene, as well as *TEGT* as a housekeeping gene. Premier3 software was used to design RT-qPCR primers with a melting temperature of 60 to 65 degrees Celsius, primer lengths of 18 to 23 nucleotides, and PCR amplicon lengths of 75 to 150 base pair as shown in Table 1

Table 1: Primers sequences**.**

Primers		Sequences 5′→3′	Annealing Temp (C)
LTB4R F	*F*	CCTGTGATAAGTCTCCTTGTTAG	60
LTB4R	*R*	GTGAAATGGAGGGAAGGAAG	
TEGT	*F*	TGCTGGATTTGCATTCCTTACA	65
TEGT	*R*	ACGGCGCCTGGCATAGA	

Gene Expression
cDNA synthesis from mRNA:

The GoTaq®1-Step RT-qPCR System kit (Promega, USA) was used to assess the expression of *LTB4R* genes. It is a one-step RT-qPCR reagent method for quantitative RNA analysis. Effective, sensitive service, and one-step linear RT-qPCR quantification across a broad range of RNA template inputs; GoScriptTM Reverse Transcriptase, and GoTaq®qPCR Master Mix

are combined in the GoTaq®1-Step RT-qPCR system. According to the instructions of the manufactured company, in a reaction volume of 20 µl, the procedure was carried out. The total amount of RNA that needed to be reversed transcribed was 4 µl.

Protocol

Reverse transcription reactions should be assembled in RNase-free environment. The RNA templates and all reagents were thawed, and each solution was mixed gently. The RT FDmix tubes were placed in a PCR tube rack. The reaction component was added to the RT FDmix tube as in Table 2.

Table 2: Reaction volume and components of reverse transcription reaction used to prepare cDNA from total RNA

Component	Volume
RT FDmix	1 tube
Total RNA	4µl
Nuclease-freeH20	up to 20µl

Tubes were placed in a thermal cycler program as in Table 3.

Table 3: Thermal cycler steps for cDNA Reverse Transcription.

	Step1	Step2	Step3	Step4
Temperature	25 ºC	42 ºC	85 ºC	4 ºC
Time	10 min	30 min	10 min	∞

Synthesized cDNA was immediately used as a template for PCR or long-term storage at -20°C.

Quantitative real-time PCR (qRT–PCR): The expression levels of the *LTB4R* gene were estimated by the reverse transcription-quantitative polymerase chain reaction (qRT-PCR) method, which is a sensitive technique for the quantification of steady-state mRNA levels. To confirm the expression of the target gene, a quantitative real-time qRT-PCR SYBR Green assay was used. Primers sequences for the *LTB4R* gene were designed in the current study and synthesized by Alpha DNA Ltd (Canada) and stored lyophilized at -23°C. The mRNA levels of endogenous control gene *TEGT* were amplified and used to normalize the mRNA levels of the tested genes. (Sequences are shown in Table (1).

Primer preparation

A primer for *LTB4R* and *TEGT* working solution was prepared from the lyophilized primers after dissolving in nuclease-free water according to the manufacturer's instructions. To make a stock solution with a concentration of 100 µM for each primer and stored at -23°C. A working solution with a concentration of 10 µM was prepared by diluting 10µL of primers stock solution in 90 µL of nuclease-free water and stored at (-23°C) until use.

Quantitative real-time PCR (qRT–PCR) run: Quantitative real-time PCR (qRT–PCR) was performed using the MIC-4 Real-time PCR System (AUSTRALIA). The gene expression levels and fold change were quantified by measuring the threshold cycle (Ct) Using the 2xqPCR Master Mix Kits components. Every reaction was done in a duplicate. The required volume of each component was calculated according to Table 4.

Table 4: Components of quantitative real-time PCR used in gene expression experiment

Components	1µl rxn
qPCR master mix	5

Nuclease free water	2
Forward Primer (10µM)	0.5
Reverse Primer (10µM)	0.5
cDNA	2

The cycling protocol was programmed for the following optimized cycles and according to the thermal profile as shown in Table5.

Table 5: Thermal profile of genes expression.

Step	Temperature	Duration	Cycle
Enzyme activation	95ºC	5 min	1
Denature	95ºC	20 sec	40
Annealing	60ºC	20 sec	
Extension	72ºC	20 sec	

Using the real-time cycler magnetic induction cycler (mic) software, the threshold cycle (CT) was calculated for each sample. All samples were run in duplicate and mean values were calculated**.** -Expression data of selected genes were normalized against the housekeeping gene. The ΔΔCt method was used as was recommended by Kenneth and Thomas for data analysis and results were expressed as folding change in gene expression [15] , which is as follows:

For each sample, the difference between the CT values (ΔCt) for each gene of the target and the housekeeping gene was calculated
ΔCt (control) =CT (gene)-CT (HKG)
ΔCt (patient) =CT (gene)-CT (HKG)
The difference in ΔCt values is represented as (ΔΔCt) for the genes of
Interest was calculated as follows:
ΔΔCt=ΔCt (patient) - ΔCt(control)
The fold-change in gene expression was calculated as follows: Fold change=$2^{-\Delta\Delta Ct}$

Statistical Analysis

Data were statistically analyzed by utilizing Sigma plot for Windows, version 12.5 Data have appeared as mean ± standard deviation [16] .

3. Result and Discussion

cDNA reverse transcription: On the second day of RNA extraction complementary DNA reverse transcription was conducted. A common primer reaction was applied since it was needed to have cDNA for LTB4R gene and housekeeping gene. The efficiency of cDNA concentration was determined through the efficiency of qPCR conducted later on. All steps were associated with perfect yield reflecting efficient reverse transcription. According to the Tm of each primer supplied in the manufactures instructions the annealing temperature of the optimal primers was calculated depending on the following equation:

- Melting Temperature (Tm) =2 (A+T) + 4 (G+C).
- Annealing Temperature (Ta) = Tm - (2-5) ºC.

The temperatures of melting for the reverse and forward primer were also calculated according to the above equation. The lowest melting temperatures of both forward and reverse primers were calculated by comparing the annealing temperature for the forward and reverse

primers. The annealing temperature for our study gene LTB4R /60 °C. Gene expression of LTB4R was determined by Real-time Polymerase Chain Reaction (RT-qPCR), the level of gene expression was adjusted to the level of a housekeeping gene and quantified by the folding (2-ΔΔCt) and ΔCt value as shown in Figures (1), (2) respectively.

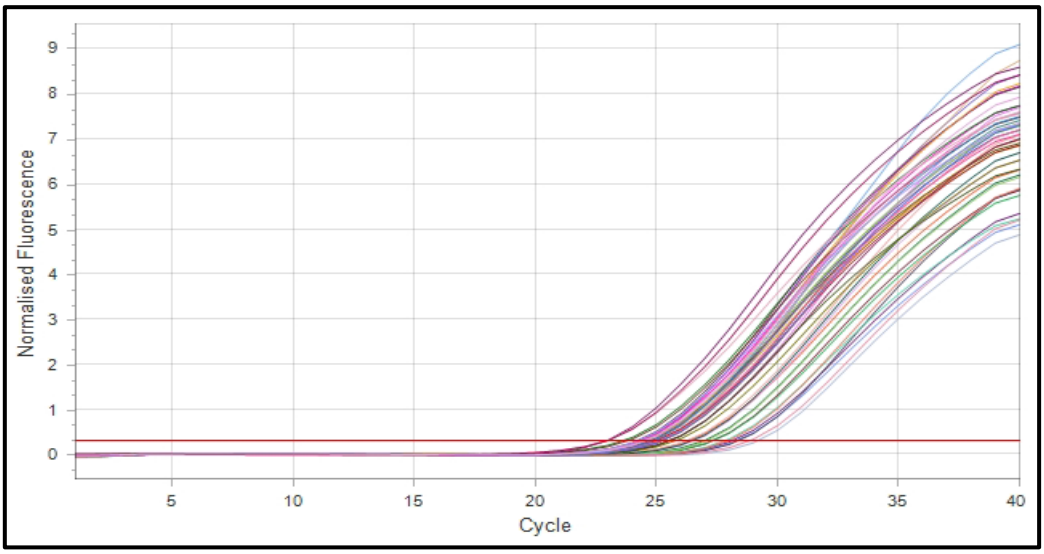

Figure 1: Amplification plots of *LTB4R gene* by magnetic induction cycler (mic) RT-qPCR.

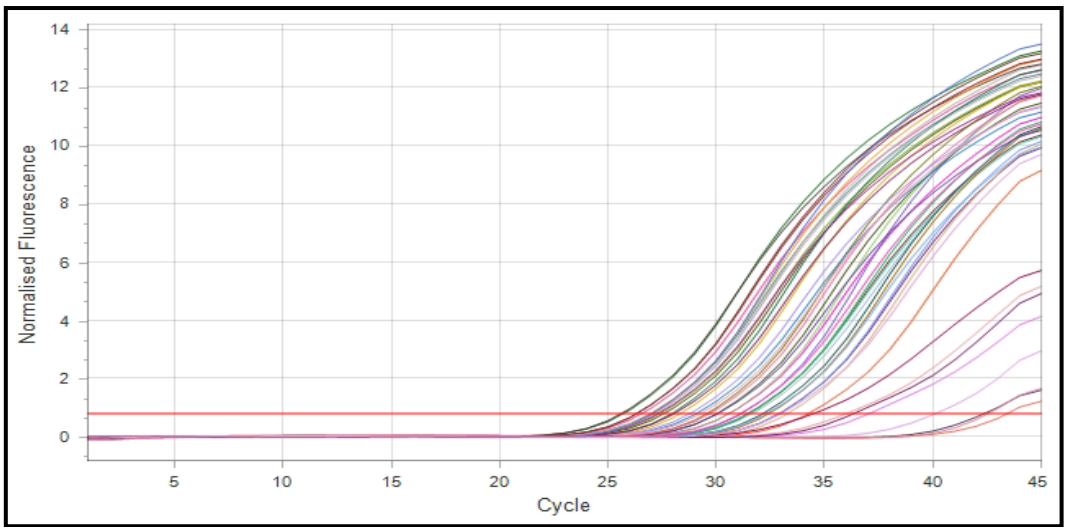

Figure 2: Amplification plots of *TEGT gene* by magnetic induction cycler (mic) RT-qPCR.

A single peak for amplicon was observed by a representative melt curve of both the LTB4R gene as well as the TEGT gene through analyzing the samples by (RT-qPCR) as shown in Figures (3), (4) such finding indicated the melt curve represented a pure single amplicon of samples and the amplification specificity was major with intercalating dye assay

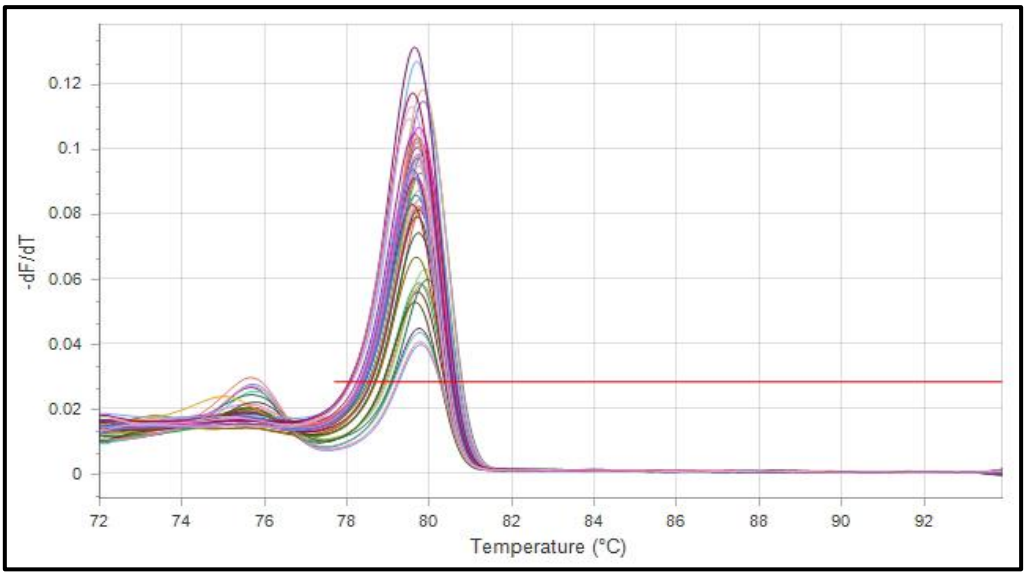

Figure 3: Melt curve of *LTB4R gene* amplicons after RT-qPCR analysis showing single peaks. Threshold 0.028 starting at 77.7 ºC.

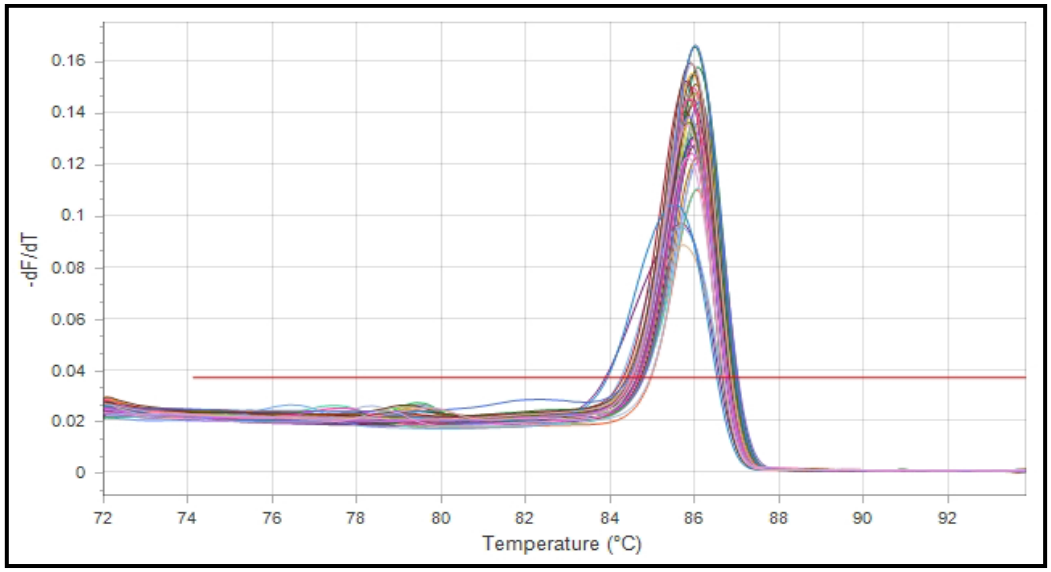

Figure 4: Melt curve of *TEGT* gene amplicons after RT-qPCR analysis showing single peaks. Threshold 0.037starting at 74.12°C

The Expression level of *LTB4R* gene in the studied groups: The expression level of LTB4R gene expression level in CML patients was higher than levels in the control group, and the ΔCt mean of LTB4R gene in blood samples of CML patients was (8.55) compared to ΔCt mean of the control group (3.29). Statistically, there was a significant increase in ΔCt mean at ($p = < 0.001$) for the LTB4R gene of the patients group compared to the ΔCt mean in the control group as shown in Table (6).

Table 6: Expression level (ΔCT) of LTB4R in CML patients and control groups

Gene expression Level ΔCT	Control (mean± SD) (n=50)	Patient (mean± SD) (n=50)	*P*-Value
LTB4R	3.29 ±1.44	8.55 ±0.468	0.001

The expression-folding mean ($2^{-\Delta\Delta ct}$) of LTB4R in the current study reveals the highest level in ($2^{-\Delta\Delta ct}$) of the LTB4R gene reached (9.12) and was significant at ($p< 0.001$) as shown in Table (7).

Table 7: Mean of LTB4R gene expression fold ($2^{-\Delta\Delta Ct}$) in patients group**.**

Genes	Gene expression ($2^{-\Delta\Delta ct}$) (mean± SD)	*P*
LTB4R	9.12 ± 4.85	0.001

According to the results of Tables (6, 7) the significant variations in gene expression levels of *LTB4R* between the Iraqi CML patients and healthy ones indicate the correlation between this gene and chronic myeloid leukemia and provide a qualified applicant to use these genes as a marker tool for detection of CML progression. These findings are supported by Sweet etal., who state that molecular changes serve as prognostic factors in CML patients in the USA [17] . Further research identified an *LTB4R* gene signature that discriminated between patients in chronic and blast phase in the USA [18] . A previous study in UK by Lucas etal., suggested that the LTB4R was significantly increased after a period of treatment in CML patients who exhibit poor response to imatinib therapy, especially at late stage indicating a higher risk of disease progression [19] . This could indicate LTB4R accumulation and a potential blockage in the arachidonic acid pathway.

In Sweden, pathways of signalling mediated by leukotrienes have been recognized as essential regulators of acute and chronic inflammation, as well as it is role in the pathogenesis of many types of cancers, including breast, lung, prostate, and colon cancers [20] . Mean of gene expression folding $2^{-\Delta\Delta ct}$ for the LTB4R gene in CML patients showed 6.90 ± 4.64 at ages (< 40 years), 10.48 ± 6.10 at ages (40-50 years), and 11.17 ± 5.02 at ages(>50 years) with no significant differences between LTB4R gene expression and ages categories. The correlation coefficient analysis for mean values of gene expression (r) of LTB4R was -0.0655, -0.0828, and -0.325 for (<40 years), (40-50 years), and (>50 years) respectively, and statistically the differences were non-significant as shown in Table (8)

Table 8: The LTB4R gene expression folding $2^{-\Delta\Delta ct}$ correlation with age categories in the CML patients group

Age categories	Number of cases (n=50)	Gene expression ($2^{-\Delta\Delta ct}$) LTB4R (mean± SD)	r	*P*
<40 years	22	6.90 ± 4.64	-0.0655	0.81
40-50 years	16	10.48 ± 6.10	-0.0828	0.79
>50 years	12	11.17 ± 5.02	-0.325	0.30

One possible interpretation of high incidence in elderly ages may be related to those younger patients transferring to doctors are less common than older ones and only when there are apparent clinical signs [21] . Also, genetic factors might affect the age distribution of CML

patients [22] . Whereas others indicate that age is not a limitation factor after treatment with TKI, as a patient in any age group can receive treatment and continue to be in better health. [23] .

The Mean of gene expression folding $2^{-\Delta\Delta ct}$ in the female patient group for LTB4R gene was 9.64±5.65, compare to the male patient group 8.46±5.38 and statistically, the differences were non-significant p>0.05 as shown in Table (9). Interestingly, our findings are similar to a study in Libya that showed a high incidence of CML in females (61%) compared to males (39%); [24] . However, our data disagreed with Melo and Barnes who reported that incidence was higher in men than in women [25] .

Table 9: Correlation of the mean of the LTB4R gene expression fold for CML patients with gender.

Gene expression $2^{-\Delta\Delta ct}$	**Patients groups**		***P***
	Female group (n=31)	Male groups (n=19)	
LTB4R	9.64±5.65	8.46±5.38	0.47

According to our study results, there was no significant correlation observed between LTB4R gene expression and gender, suggesting that CML cells have a lower rate of apoptosis and disease progression this was supported by the elevated LTB4R gene expression due to BCR-ABL protein expression. Therefore, we can conclude that the correlation between these genes expression and patients' gender might depend on the gene type. These results reflect that CML disease could affect both males and females at any age. The Philadelphia chromosome creates a hybrid BCR-ABL gene, which in turn is transcribed into chimeric BCR-ABL messenger RNA. The fusion messenger RNA is translated into p210 BCR-ABL a chimeric 210-kDa protein [26] . Therefore, diagnosis can be made based on the BCR-ABL gene by identification of the Philadelphia chromosome to confirm diagnosis as well as the characteristics of blood parameters represented by blood count and hemoglobin [27] as shown in Table (10), which revealed the mean and standard deviation for each parameter in CML patients. Data exhibited that the means value for WBC (148.99 x 10^9), RBC (3.83 x 109), platelets (442.6 x 10^9), hemoglobin (11.13) and BCR-ABL gene (41.32).

Table 10: Mean of the clinical parameters in CML patient groups

Parameters	**Means (n=50)**	**SD**
WBC x 10^9/L	148.99	89.63
RBC x 10^9/L	3.83	0.64
Platelets x10^9/L	442.6	257.39
Hemoglobin g/L	11.13	2.11
BCR-ABL gene	41.32	14.92

Correlation coefficient analysis for mean values of the following parameters in respect to *LTB4R* gene expression was positive 0.131 for WBC Moreover, the differences were statistically non-significant.RBC was 0.021 and the differences were statistically non-significant. Platelets were -0.062 and the differences were statistically non-significant. Hemoglobin was 0.015 and the differences were non-significant. BCR-ABL gene was 0.210 and the differences were statistically non-significant (Table 11). A Positive correlation between

the BCR-ABL gene and LTB4R gene expression demonstrated the correlation between the LTB4R gene and WBC in CML patients was represented in figure (3) and the correlation between the LTB4R gene expression and BCR-ABL gene in CML patients was represented in figure (4).

Table 11: The correlation analysis of LTB4R gene expression with clinical parameters in CML patients groups

Parameters	LTB4R	
	r	*P*
WBC x 10^9/L	**0.131**	0.365
RBC x 10^9/L	0.021	0.882
Platelets x10^9/L	-0.062	0.668
Hemoglobin g/L	0.015	0.917
BCR-ABL gene	**0.210**	0.144

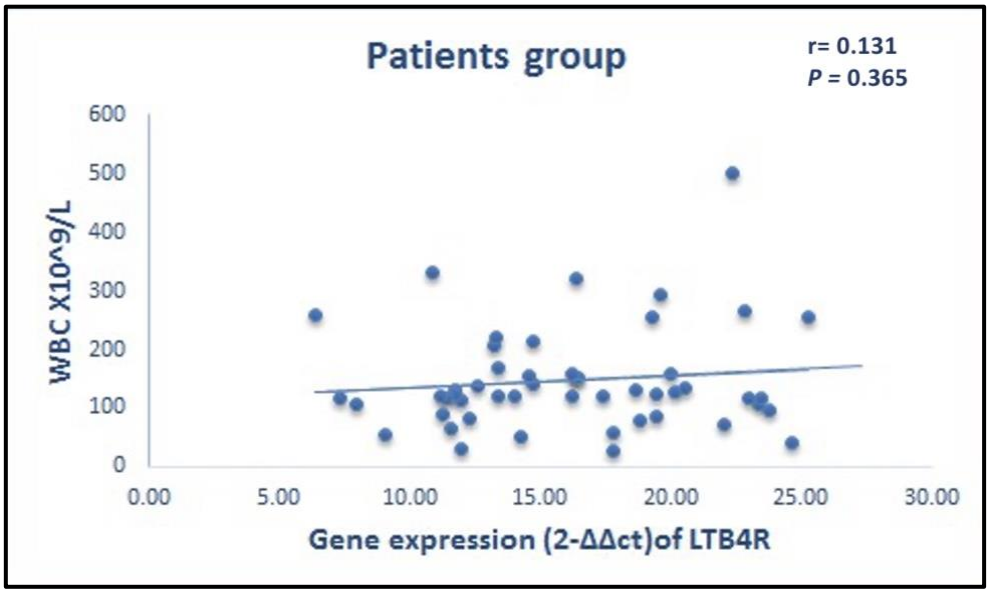

Figure 3: The association between WBC and LTB4R gene expression in the patients group.

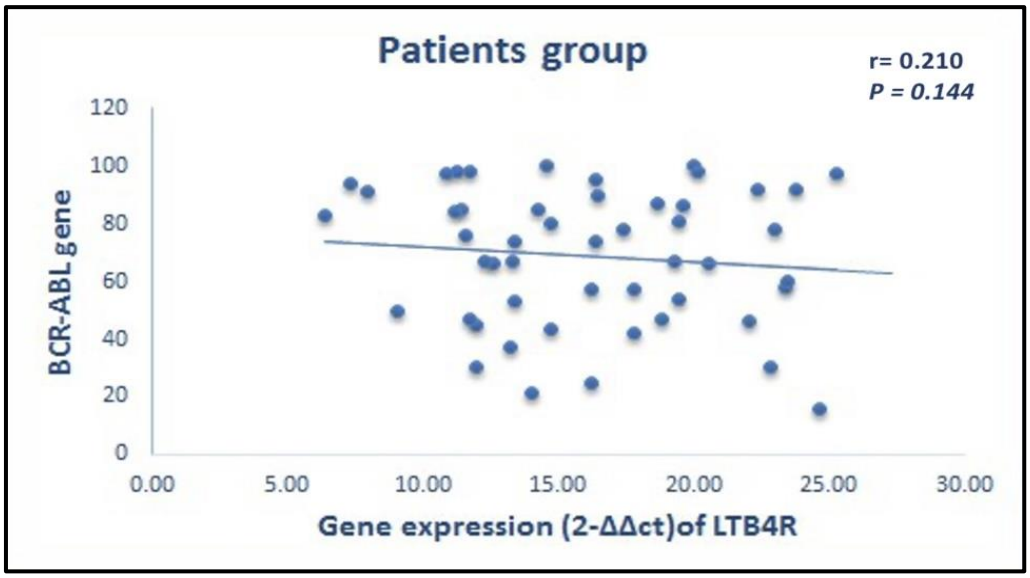

Figure 4: The association between BCR-ABL gene and LTB4R gene expression in the patients group.

LTB4R gene expression was measured at the chronic phase of CML patients to see if there was a relationship between the gene expression levels and the clinical outcome, LTB4R levels were increased, indicating that the arachidonic acid pathway is working normally up to the point of LTB4R production [19] . A correlation was found between neutrophilic granulocytes and metamyelocytes and the amount of LTB4R formed r = 0.600, P less than 0.05. a higher peripheral WBC count in CML is associated with a worse prognosis. In CML, patients variations in LTB4R observed may be due to leukemia rather than the physiological response to therapy [28] . Our results suggest the possibility of applying molecular techniques including (RT-qPCR) that LTB4R gene expression as a biomarker in CML patients. Oehler et al., confirmed these results by suggesting 6 genes (NOB1, DDX47, IGSF2, LTB4R, SCARB1, and SLC25A3) as beneficial biomarkers in patients very early on the timeline of the CML progression. At the time of diagnosis, gene expression profiling may be useful for identifying patients with a poor prognosis and elucidating the biological basis of CML disease progression [29] .

Conclusion:

Our results concluded that a non-significant positive correlation between LTB4R gene expression level with both the BCR-ABL gene and WBC and the *LTB4R* gene expression level could act as a marker for the prognosis of CML.

Ethics

Ethical clearance

This research was ethically approved by the Research Ethical Committees of the Ministry of Environmental and Health and the Ministry of Higher Education and Scientific Research, Iraq.

Conflict of interest

The authors declare that they have no conflict of interest.

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