CORRELATION STUDY BETWEEN THREE DIFFERENT GENES EXPRESSION AND CHRONIC MYELOID LEUKEMIA IN IRAQ

H. M. Aljoubory * **M. F. Altaee***
Researcher **Assis. Prof.**
***Department of Biotechnology / College of Science / University of Baghdad / Iraq.**
e.mail: maha. fakhry@sc.uobaghdad.edu.iq

ABSTRACT

The following study was conducted to investigate the correlation between the expression of three different genes (*NOB1, DDX47, CD101*) with the occurrence and development of chronic myeloid leukemia (CML) in Iraq. The difference in the expression of these genes between patients and healthy controls was studied. Moreover the correlation of age and gender with CML occurrence and comparing with control was also examined. Results showed significant increases in mean of gene expression level (ΔCt) of patient groups for all genes compared to the corresponding ΔCt means in control group, also the gene expression folding ($2^{-\Delta\Delta Ct}$) reflect significant differences in the expression of these genes and CD101, mRNA showed the highest level in CML patients which reached to (3.44), while NOB1 and DDX47 recorded (2.90 and 1.08) respectively. On the other hand no significant differences were recorded according to age and gender between CML patients and control, CML disease could affect any age and both male and female.

Key wards: *NOB1, CD101, DDX47,* pateints, age, gender.

مجلة العلوم الزراعية العراقية -2021 :52 (3):611-619 الجبوري والطائي

دراسه الارتباط بين التعبير الجيني لثلاث جينات مختلفه وابيضاض الدم النقوي المزمن في العراق

هدير مثنى الجبوري **مها فخري الطائي**
باحث **استاذ مساعد**

المستخلص

تم اجراء هذه الدراسه للتحري عن العلاقه بين التعبير الجيني لثلاث جينات مختلفه (*NOB1, DDX47,CD101*) وحدوث وتطور مرض ابيضاض الدم النقوي المزمن في العراق. ايضا تم دراسه الفرق في التعبير الجيني للجينات المذكوره سابقا بين المرضى ومجموعه السيطرة الاصحاء. اضافه الى ذلك تم فحص علاقه العمر والجنس بحدوث ابيضاض الدم النقوي المزمن ومقارنته مع السيطرة. اظهرت النتائج وجود ازدياد معنوي في متوسط التعبير الجيني للمرضى ولكل الجينات بالمقارنه مع السيطرة وايضا سجلت النننتائج وجود فروقات معنويه في متوسط تعبير الاضعاف الجبني للجينات في حين اظهر جين CD101 اعلى متوسط في التعبيرفي مرضى ابيضاض الدم النقوي المزمن حيث بلغ (3.44) بينما سجل كل من NOB1 و DDX47 (2.90 و 1.08) على التعاقب. من الناحيه الاخرى لم تضهر النتائج وجود فروقات معنويه بين المرضى والاصحاء نسبه الى العمر والجنس وان مرض ابيضاض الدم النقوي المزمن يمكن اي يصيب اي عمر وكل من الذكور والاناث.

الكلمات المفتاحيه: *NOB1, CD101, DDX47*, مرضى, عمر , جنس.

Received:22/6/2020, Accepted:6/9/2020

INTRODUCTION

As a myeloproliferative disorder, chronic myeloid leukemia (CML) characterized by enhanced myeloidic cell proliferation. The disease progresses through different phases: chronic, accelerated and undifferentiated blast crises. A cytogenetic abnormality is the charestristics of CML consisting of a reciprocal translocation between the q arms of chromosomes 9 and 22, t(9;22): the chromosome in Philadelphia (7). The oncogene abl being transferred from the q arm of chromosome 9 into the q arm of chromosome 22 in the bcr region as a result of this translocation and a chimeric protein with heavy activation of the tyrosine kinase was encoded by the fusion gene bcr/abl (1). This work laid the foundation for the discovery of a tyrosine kinase inhibitor (TKI) as CML therapy (3). Hydroxyurea and eventually interferons and allogeneic transplantation of stem cells were used as effective treatment. However the development of imatinib and other drugs has changed the concept of disease treatment (10). CML patients commonly have few symptoms, common symptoms include abdominal fullness due to splenomegaly, anemia-related fatigue, bone pain associated with a packed bone marrow, bleeding, weight loss, malaise or night sweats. An enlarged spleen is often visible during physical examination whereas an enlarged liver or purpura is less normal (13).

***NOB1*:** Nin one binding 1 human gene is composed of nine exons and eight introns, and is located on chromosome 16q22.1 and it give RNA-binding protein NOB1 (19). Ribosome assembly required over 210 protein and RNA. The modification and cleavage of the initial rRNA, folding of the rRNA, and binding of ribosomal proteins and 5S RNA was mediated by these factors (17). However, pre-rRNA processing required the involving of NOB1. NOB1 cleaves 20S rRNA intermediate at cleavage site D to produce the mature 18S rRNA in a late cytoplasmic processing step. Abnormal expression of NOB1 in leukemia has been detected (6).

***DDX47*:** DDX47 (DEAD box polypeptide 47) is a member of the DEAD box family of proteins. Many conserved motifs which include the highly conserved DEAD (Asp-Glu-Ala-Asp) amino acid sequence motif were conserved by this family. The function as ATP-dependent RNA helicases is the major activity of DEAD box proteins (20). All aspects of RNA metabolism and function which include pre-mRNA splicing, RNA synthesis, RNA degradation, RNA export, RNA translation, RNA secondary structure formation, ribosome biogenesis, and the assembly of RNP complexes were mediated by DEAD proteins as helicases. DDX47 can transfer between the cytoplasm and the nucleus, with an RNA-independent ATPase activity. DEAD box RNA helicases are located on chromosome 12p13.1 and it is the key components of life (9).

***CD101*:** (Cluster of Differentiation 101) is a protein coding of the gene located on chromosome 1p13.1 (18). However, inhibition of T-cells proliferation induced by CD3, inhibition of expression of IL2 on activated T-cells and secretion of it, inhibition of tyrosine kinases that are required for IL2 formation and cellular proliferation are one of the roles of CD101. It may also be a marker of $CD4^+$ and $CD56^+$ leukemic tumor cells (12). The following study was aimed to investigate the correlation between the expression of three different genes (*NOB1, DDX47, CD101*) with the occurrence and development of chronic myeloid leukemia (CML).

MATERIALS AND METHODS

Patients and Controls : In this study forty-four CML patients with an age ranging between (19-70) years old that were divided a according to gender into (19) male and (25) female were obtained from medical city (Baghdad Teaching Hospital/ Baghdad/Iraq), in addition to forty-six healthy controls with an age ranging between (19-62) years old and that were divided a according to gender into (25) male and (21) female. The characteristic peripheral blood smear analysis and complete blood profiling along with bone marrow examination data of the patients is the basis for diagnosis of CML.

Blood collection: one ml blood sample was taken from each patient and healthy control and collected in TRIzol TM Reagent containing tube for RT-qPCR analysis.

Total RNA Extraction with TRIzol: RNA was isolated from sample according to the

protocol of TRIzolTM Reagent as the following steps:

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

For three phase's separation: 0.2 mL of chloroform was added to the lysate for each tube, then the tube cap secured. All mixes were Incubate for 2–3 minutes then centrifuge for 10 minutes at 12,000 rpm. Then, the mixture was separated into a lower organic phase, interphase, and a colorless upper aqueous phase. The RNA containing aqueous phase was transferred into a another clean tube.

For RNA precipitation: to the aqueous phase, isopropanol (0.5 ml) was added and incubated for 10 minutes then centrifuge for 10 minutes at 12,000 rpm. Total RNA was precipitate formed a white gel-like pellet at the bottom of the tube. Supernatant was then discarded.

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

For 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 were determined by spectrophotometer nano drop (Q5000 (UV-VIS), the concentration is measured in ng /µl unit, and the purity is measured by optical density (OD) ratio 260 / 280nm (DNA and protein absorption wave length). The accepted purity of RNA is 1.7- 2. According to the lowest concentration of samples the concentration of RNA samples was normalized by using the following equation:

$Vn = Co*V0 / Cn$

Where, Co is the lowest concentration, VO is the normalized volume (equal to 100 µL, Cn current sample concentration and Vn is the volume of current sample that will diluted by T.D.W to generate totally 100 µl.

Primer design: The cDNA sequences of *(NOB1, DDX47, CD101* and *TEGT*) genes were obtained from the NCBI GenBank database. RT-qPCR primers were designed using Primer Premier 3 software with melting temperature between 60 to 65 C, primer length between 18 to 23 nucleotides, and PCR amplicon length within 75 to 150 base pair as shown in Table 1.

Table 1. Primers sequences

Primer Name	Sequence	Annealing Temperature (C)
NOB1-F	5`-CATACCAGTTGGAAGCAGAG-3`	60
NOB1-R	5`-GGCAGATGGAAACCAGAAA-3`	
DDX47-F2	5`-CACCAAGATCCAGATTGAAGC-3`	
DDX47-R2	5`-TAGAATGGGCAAAGCAAAGG-3`	
TEGT-F	5`-TGCTGGATTTGCATTCCTTACA-3`	
TEGT-R	5`-ACGGCGCCTGGCATAGA-3`	
CD101-F2	5`-GGCATCTTTCTTTCTCCTTCT-3`	65
CD101-R3	5`-GGGTAACCTTCAGCTCTAAAC-3`	

Gene Expression

cDNA synthesis from mRNA: The kit that was used to assess the expression of (*NOB1, DDX47,* and *IGSF2*) genes was GoTaq®1-Step RT-qPCR System (Promega, USA). It is a reagent system for quantitative analysis of RNA using a one - step RT- qPCR protocol. According to the manufacturer instructions, the procedure was performed with a 20 µl reaction volume. To be reversely transcribed the total RNA volume was (4µl).

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 on 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(µl)\Reaction
RT FDmix	1 tube
Total RNA	4 µl
Nuclease-free H2O	up to 20 µl

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

Table 3. Thermal cycler steps of conditions cDNA Reverse Transcription

	Step 1	Step 2	Step 3	Step 4
Temperature	20 ºC	40°C	80 ºC	4 ºC
Time	10 min	30 min	5 min	∞

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

Quantitative Real Time PCR (qRT–PCR):

The expression levels of *(NOB1, DDX47,* and *CD101*) genes 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 target gene, quantitative real time qRT-PCR SYBR Green assay was used. Primers sequences for *NOB1*, *DDX47*, and *CD101* genes were designed in the current study and synthesized by Alpha DNA Ltd (Canada) and stored lyophilized at (-23°C). Primers sequences are shown in Table (1). The mRNA levels of endogenous control gene *TEGT* were amplified and used to normalize the mRNA levels of the tested genes. TEGT primers sequences are also shown in Table (1).

Primer preparation

A primer for *(NOB1*, *DDX47*, *CD101* and *TEGT)* working solution were prepared from the lyophilized primers after dissolving in nuclease free water according to the manufacture, to make a stock solution with a concentration of 100 µM for each primers 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) employing the 2xqPCR Master Mix Kits components. Every reaction was done in a duplicate. The required volume was calculated according to Table 4.

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

components	1 µl rxn
qPCR master mix	5
Nuclease free water	2
Forward Primer (10 µM)	0.5
Reverse Primer (10 µM)	0.5
cDNA	2

The cycling protocol was programed for the following optimized cycles and according to the thermal profile as shown in Table 5

Table 5. Thermal profile of genes expression

Step	Temp.	Duration	Cycles
Enzyme activation	95ºC	5 min	1
Denature	95ºC	30 sec	40
Annealing	60ºC	30 sec	
Extention	72C	30 sec	

Using the real-time cycler software, the threshold cycle (CT) was calculated for each sample. All samples were run in duplicate and mean values were calculated (Appendix 3)**.** Expression data of selected genes were normalized against housekeeping.The ΔΔCt method by (5) was used as was recommended for data analysis and results were expressed as

folding change in gene expression as follow: For each sample, the difference between the CT values (ΔCt) for each gene of target and the housekeeping gene was calculated

ΔCt (control)=CT(gene)-CT(HKG)

ΔCt (patient)=CT(gene)-CT(HKG)

The difference in ΔCt values(ΔΔCt) for the genes of interest was calculated as follow:

ΔΔCt=ΔCt(patient)- ΔCt(control)

The fold-change in gene expression was calculated as follow:

Fold change = $2^{-\Delta\Delta Ct}$.

Statistical analysis

Data were statistically analyzed by utilizing SPSS for Windows, version 17(SPSS Inc. Chicago, IL, United States). Data were appeared as mean ± standard deviation (2).

RESULTS AND DISCUSSION

Quantitative Expression of NOB1, CD101 and DDX47 genes: Total RNA was successfully extracted from all samples. The concentration of total RNA ranged from 72 to 193 ng/ µl. While it range from 87 to191 ng/µl in healthy group. The purity of total RNA samples ranged from 1.82 to1.92 while with control group it recorded form 1.79 to1.99.

cDNA reverse transcription: Complementary DNA reverse transcription was conducted on the second day of RNA extraction. A common primer reaction was applied since it was needed to have cDNA for all the genes in the study and housekeeping gene. The efficiency of cDNA concentration was assessed through the efficiency of qPCR conducted later on. All steps were associated with perfect yield reflecting efficient reverse transcription. Optimal primers annealing temperature was calculated from the Tm of each primer supplied in the manufactures instructions according to specific equations. The annealing temperature was calculated according to the following equation, which requires the sequence of the primer because the amounts of specific nucleotides are needed. The equation as follow:

Melting Temperature (Tm) =2 (A+T) + 4 (G+C).

Annealing Temperature (Ta) = Tm - (2-5) ºC

Using the above equations, the temperatures of melting for the reverse and forward primer were calculated. The lowest temperature (ºC) was chosen by comparing the annealing temperature for forward and reverse primers (11). Quantitative expression of *NOB1*, *CD101* and *DDX47* genes and housekeeping gene *TEGT* were determined by Real Time Polymerase Chain Reaction, in which the relative quantitation method was employed. The gene expression level was normalized to the level of a housekeeping gene and quantified by the ΔCt value and folding ($2^{-\Delta\Delta Ct}$) method as shown in Figures (1, 2, 3, 4) respectively.

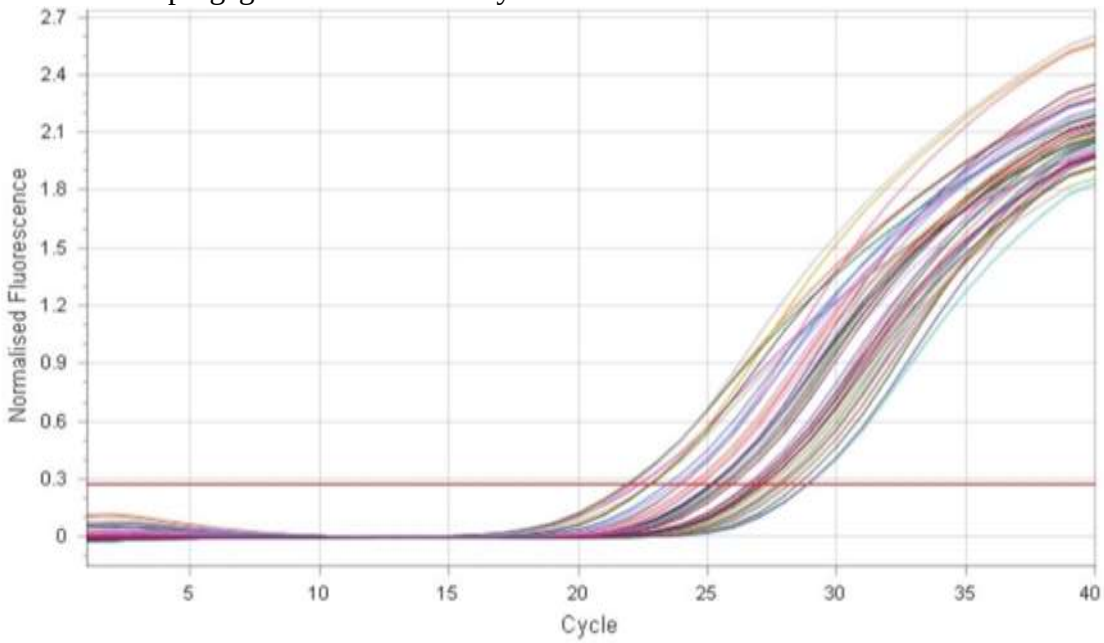

Figure 1. Amplification plots of *NOB1* gene by RT-PCR.

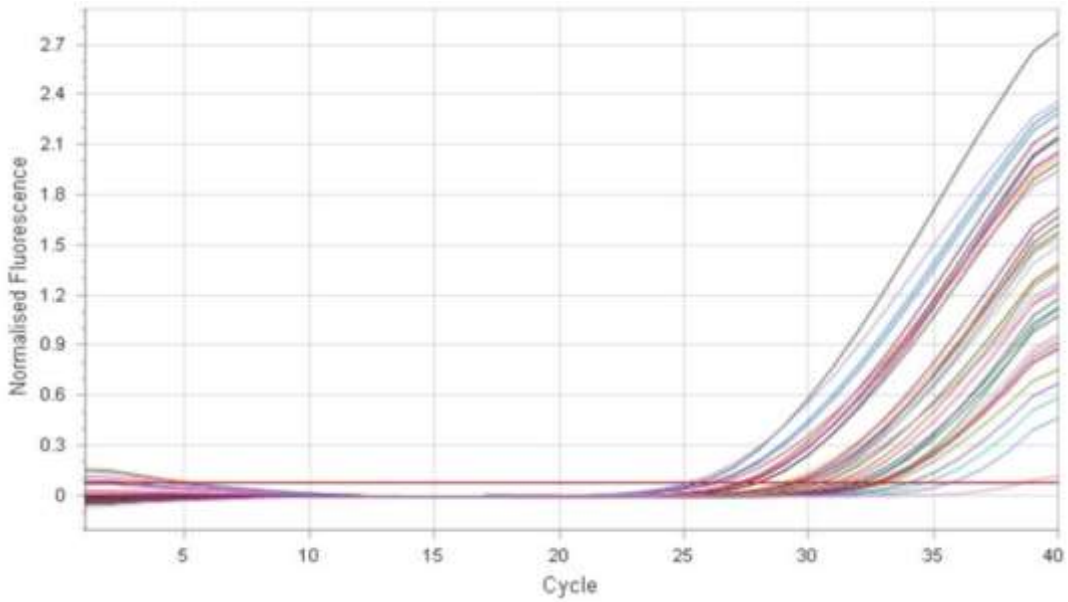

Figure 2. Amplification plots of *CD101* gene by RT-PCR

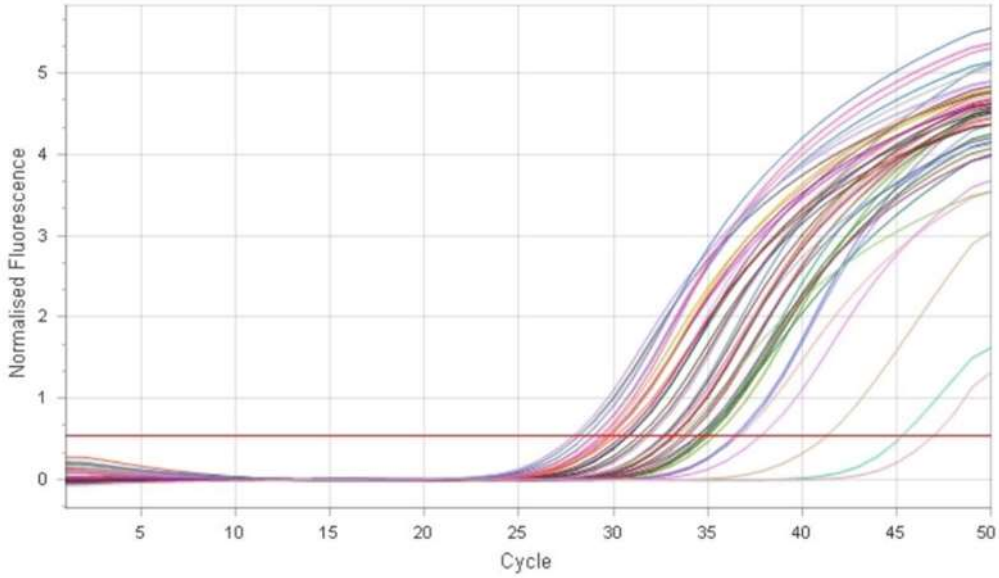

Figure 3. Amplification plots of *DDX47* gene by RT-PCR

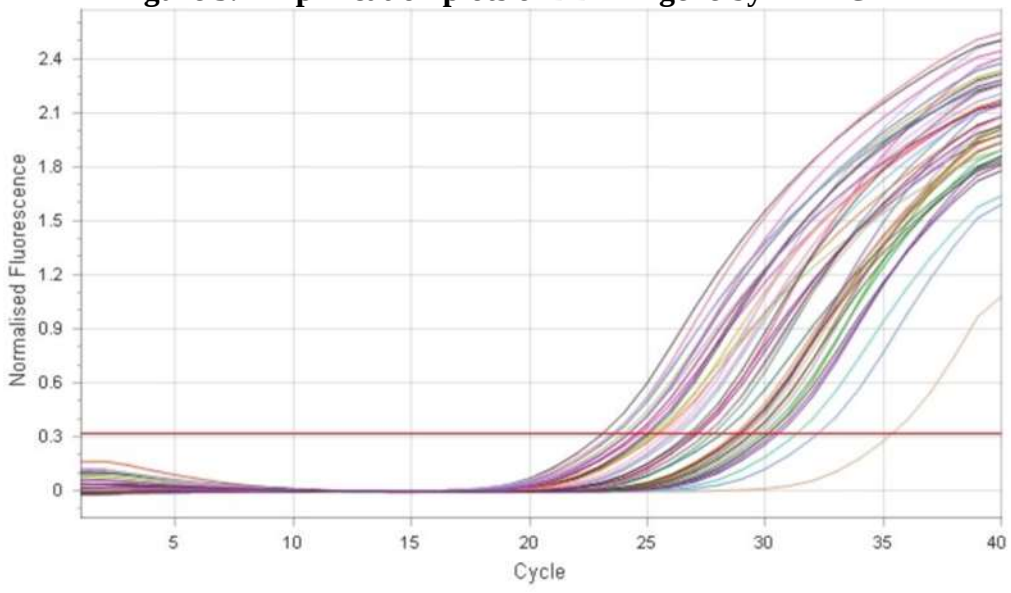

Figure 4. Amplification plots of *TEGT* gene by RT-PCR

A representative melt curve *NOB1* gene for samples analyzed by RT-PCR is given in Figure 5, in which, a single peak was observed for the amplicons. Such findings are interpreted that the melt curve represented a pure, single amplicon for each sample, and the specificity of amplification was considered to be great with intercalating dye assay.

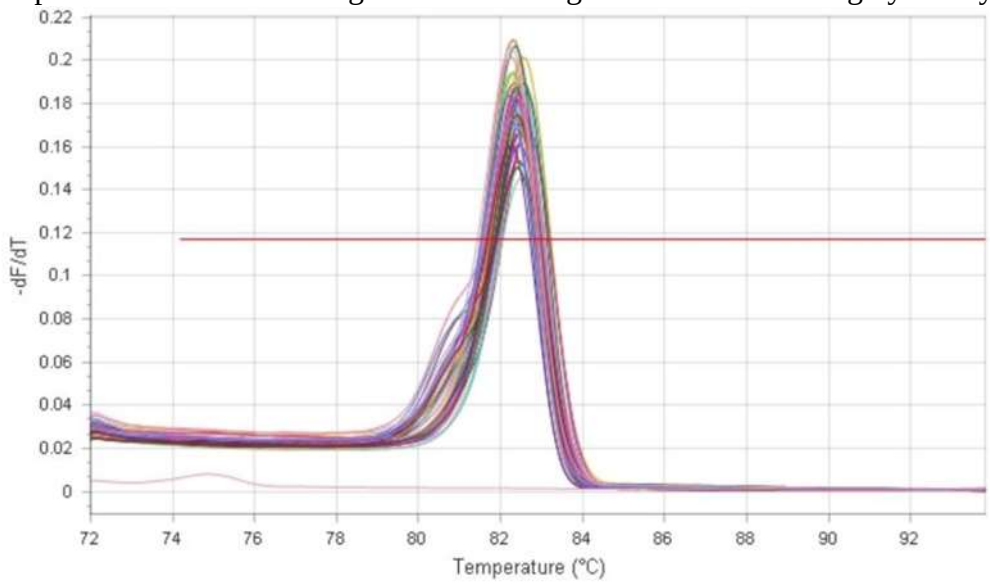

Figure 5. Melt curve of *NOB1* gene amplicons after RT-qPCR analysis showing single peaks

Expression level of *NOB1, CD101,* and *DDX47* genes in the studied groups: The ΔCt mean of *NOB1* gene in blood samples of CML patients was (21.90), CD101 ΔCt mean was (23.44) and for DDX47 ΔCt mean was (18.08). There was a significant $p<0.05$ increases in ΔCt mean of patient groups for all genes compared to the corresponding ΔCt means in control group as shown in Table 6.

Table 6. Expression level (ΔCt) of *NOB1, DDX47,* and *CD101* in patients and control groups

Gene expression Level ΔCt	Control (n=46) (mean ± SD)	Patients (n=44) (mean ± SD)	*P*
NOB1	**7.79±0.32**	**21.90±1.43**	**0.01**
DDX47	**5.67±0.27**	**18.08±0.40**	**0.02**
CD101	**7.62±0.27**	**23.44±1.64**	**0.00**

However, the expression folding ($2^{-\Delta\Delta Ct}$) of genes reflect significant differences in the expression and CD101 mRNA showed the highest level in CML patients which reached to 3.44, while NOB1 and DDX47 recorded 2.90 and 1.08 respectively as shown Table 7.

Table 7. Mean of gene expression ($2^{-\Delta\Delta Ct}$) of NOB1, DDX47, and CD101 mRNA in patients group

Genes	Gene expression ($2^{-\Delta\Delta Ct}$) (mean ± SD)	ANOVA *P* value
NOB1	**2.90±1.43[a]**	
DDX47	**1.08±0.40[b]**	**0.01**
CD101	**3.44±1.64[a]**	

Different small letters denote significant differences
Similar small letters denote non-significant differences

The expression of genes showed significant variation between the CML patients and control. Such findings are consistent with previous study (10, 16), which generally reported the correlation of the high percentage of expressing these genes in CML patients. The current study highlighted the importance of these genes in occurrence and development of chronic myeloid leukemia and results clearly indicate an involvement of all the studied genes in the CML invasion and metastasis. Since the reason for high expression of previous genes in CML patients is presently unclear but may be related to disturbances in this type of cancer leading to anomalies in the production of these genes. The correlation of these genes with other types

of cancer was also recorded by some researches (14 , 17). In conclusion, NOB1, CD101 and DDX47 mRNA expression were significantly upregulated in CML tissue, in comparison with controls. These results suggest that increased genes expression might be a useful diagnostic marker and might also become a potential target in the treatment of chronic myeloid leukemia in Iraq.

Correlation of age and gender with CML and control groups: result revealed in Table 9 and 10 relation of age and gender with CML in comparison with control. Results showed that no significant differences p> 0.05 were recorded for both age and gender between CML patients and controls, similar results were also obtained by (4, 8). The percentage of patient male was 21.11% while control male record 27.78%. Moreover patients female showed 27.78% percentage while control female showed 23.33% percentage as shown in Table 8. Also the mean patient's age was 40.36 while the mean control age was 41.00 as shown in Table 9. These findings suggest that CML can occur in both male and female and can affect all ages.

Table 8. Frequencies of control and patients groups depending on gender

Groups	Gender	Proportions	*P*
Patients (n=44)	**Male**	**19(21.11%)**	**0.28**
Control (n=46)	**Male**	**25(27.78%)**	
Patients (n=44)	**Female**	**25(27.78%)**	
Control (n=46)	**Female**	**21(23.33%)**	

Table 9. Mean of age parameter in control and patients groups

	Control (n=46)(mean ± SD)	Patients (n=44)(mean ± SD)	*P*
Age (years)	**41.00±12.50**	**40.36±12.73**	**0.81**

REFERENCES

1. Deininger, M. 2008. Chronic myeloid leukemia: An historical perspective. Journal of pediatric Oncology. 2(3), 418

2. Forthofer, R. N. and E. S. Lee 2014. Introduction to biostatistics: a guide to design, analysis, and discovery. Elsevier

3. Gupta, A. 2009. Successful use of terbutaline in persistent priapism in a 12-year-old boy with chronic myeloid leukemia. Pediatric hematology Oncology. 26: 70-83

4. Jonathan Cowman, Eimear Dunne, Irene Oglesby, Barry Byrne, Adam Ralph, Bruno Voisin, Sieglinde Mullers, Antonio J. Ricco and Dermot Kenny 2015. Age-related changes in CML are more profound in women than in men, Journal of Nature, 10:1038.

5. Kenneth, J. and M. Thomas 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2^{-DDCT} method. Methods. 25: 402–408

6. Kun Liu, Hong-Lin Chen, Shuo Wang, Ming-Ming Gu, Xin-Ming Chen, Shuang-Long You 2016. High expression of RIOK2 and NOB1predict human non-small cell lung cancer outcomes. Journal of Nature. 6(28): 666-680.

7. Mahmood S., B. Hamdan and S. Al-Mayah 2019. Association of BCR-ABL transcript variants with different blood parameters and demographic features in Iraqi chronic myeloid leukemia patients. Journal of Molecular Genetics and Genomic Medicine. 10:1002-1021.

8. Marijke G., J. Maxwell, C. Steffen and S. Milhelm 2018. Comparisons of blood parameters, red blood cell deformability and circulating nitric oxide between males and females considering hormonal contraception :A longitudinal gender study. Journal of Frontiers in Physiology. 9:1835.

9. Moumita S. and K. Ghosh 2016. DEAD box helicase: crucial regulators of gene expression and oncogenes, Journal of Frontiers in Bioscience, 21:225-250

10. O´Brien S. G., F. Guilhot and J. M. Goldman 2010. Chronic myeloid leukemia. Journal of Pediatric Science. 2(3): 24

11. Patel, N. and N. Prakash 2013. Principle and Tools for Primer Design. Atmiya. Spandan. 1: 79 -95

12. Rivas A., C. L. Ruegg, J. Zeitung, R. Laus, R. Warnke, C. Benike and E. G. Engleman 1995. A novel leukocyte surface protein that participates in T cell activation. Tissue

distribution and functional studies. J. Immunol. 154:4423-4433

13. Sandip Kumar, Vikas Kumar Gupta, Anju Bharti, Vineeta Gupta and Jyoti Shukla 2019. A study to determine the clinical, hematological, cytogenetic, and molecular profile in CML patient. Indian Journal of Family Medicine and Primary Care. 8(7), 2450-2455

14. Shan, L., M. Wei, Z. Wei and L. Jia 2013. Expression of the NOB1 gene and its clinical significance in papillary thyroid carcinoma. Journal of International Medical Research. 41(3): 568–572

15. Ulrich Mahlknecht and Simone Kaiser 2017. Age-related changes in peripheral blood counts in CML. Journal of Experimental and Therapeutic Medicine. 1(6): 1019-1025.

16. Vivan, G., Y. Ka, and G. Yongjae 2019. The derivation of diagnostic markers of chronic myeloid leukemia progression from microarray data. Blood. 114: 3292–3298

17. Xiao-Wen He, Tao Feng, Qiao-Ling Yin, Yuan-Wei Jian and Ting Liu 2015. NOB1is essential for the survival of RKO colorectal cancer cells. World Journal of Gastroenterology, 21(3), 868-877.

18. Xochen Mattner 2018. Genetic and functional data identifying *Cd101* as a type 1 diabetes (T1D) susceptibility gene in nonobese diabetic (NOD) mice. Plos. Genetics. 14: 1-23

19. Yuanhui W., Y. Y. Jianhua and L. Xiaobo 2018. Effects of NOB1 on the pathogenesis of osteosarcoma and its expression on the chemosensitivity to cisplatin. Oncology Letters. 15(3): 3548–3551

20. You, K. T., J. Park and V. Narry 2015. Role of the small subunit processome in the maintenance of pluripotent stem cells. Gene and Development. 29: 2004-2009.