CASPASE 9 GENE EXPRESSION AND CASPASE 9 PROTEIN IN CHRONIC MYELOID LEUKEMIA IN IRAQ

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ABSTRACT

The following study was carried out to investigate gene expressions of the CASPASE 9 gene and CASPASE 9 protein with the development of chronic myeloid leukemia (CML) in Iraq. The differences in the expression of this gene between patients and healthy controls were included. The correlation of age and gender with CML occurrence comparing with controls was studied. Results showed significant increases in the mean of gene expression level (ΔCt) of patient groups compared to the corresponding ΔCt means in control group, also the gene expression folding ($2^{-\Delta\Delta Ct}$) of CASPASE 9 gene reflect significant differences in the expression showed the highest level in CML patients which reached to (16.19), the level of CASPASE 9 protein was (1.025 ng/ml) in patients with CML compared to control groups with significant differences. On the other hand no significant differences were exhibited according to age and gender between CML patients and control, this result suggest that CML can affect all ages and occur in both male and females.

Key words: patients, age, gender, healthy, chronic myeloid

التعبير الجيني للكاسبيز 9 وبروتين الكاسبيز وابيضاض الدم النيقاني المزمن في العراق

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المستخلص

أجريت هذه الدراسة للتعرف على التعبير الجيني للكاسبيز ٩ وكذلك بروتين الكاسبيز ٩ في تطور مرض ابيضاض الدم النيقاني المزمن في العراق وتم دراسة الفروقات في التعبير الجيني لهذا الجين بين المرضى ومجموعة السيطرة الاصحاء ودرست العلاقة بين عمر وجنس المرضى بحدوث ابيضاض الدم النيقاني ومقارنته مع السيطرة. أظهرت النتائج وجود زيادة معنوية في متوسط التعبير الجيني للمرضى بالمقارنة مع السيطرة ويظهر التعبير الجيني للكاسبيز ٩ فروقات معنوية في متوسط التعبير الجيني للمرضى وبمستوى عالي وصل الى (16.19) وكان متوسط تركيز بروتين الكاسبيز ٩ (١،٠٢٥) نانوغرام/ مللتر في مرضى ابيضاض الدم النيقاني مقارنة مع مجموعة السيطرة مع وجود فروقات معنوية .من جهة أخرى لم تظهر فروقات معنوية بين المرضى والاصحاء تبعا الى العمر والجنس .وتفترض النتائج ان مرض ابيضاض الدم النيقاني المزمن يمكن ان يصيب كافة الاعمار ويحدث في كلا الجنسين الذكور والاناث.

الكلمات الرئيسية: المرضى، العمر، الجنس، الأصحاء ,ابيضاض الدم النيقاني.

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INTRODUCTION

CML is a chronic myeloproliferative disorder with an initially chronic course lasting for 3–5 years, this proliferation of mutant stem cell will generate some differentiated cells leading to an expending in the total myeloid mass (5) The causes of CML are essentially unknown and the risk factor is exposure to ionizing radiation, chemicals such as benzene (25,26). An increased prevalence of other malignancies and autoimmune diseases were found prior to CML diagnosis, indicating a genetics or acquired increased predisposition to cancer and autoimmunity in CML patients(9) CML is one of the chronic myeloproliferative disorders, resulting from a characteristic Philadelphia chromosomal abnormality, t (9; 22) (q34q11). The molecular consequence of this chromosomal translocation is production of a hybrid BCR-ABL. A genetic change takes place in an immature myeloid cell, which make red blood cells, platelets, and most types of white blood cells except lymphocytes (22). CML frequently affects adults but very rarely in children (7). Apoptosis is a natural cell death mechanism. It plays a crucial role in development and homeostasis in long-lived mammals. It is the process of programmed cell death that occurs in response to environmental stimuli. Regulation of apoptosis is important for normal growth and homeostasis, during development, embryogenesis, and modification of normal tissues, also cancer treatment. Disorders of abnormal induction of apoptosis can cause severe outcomes. Impaired regulation of apoptotic mechanisms provides an opportunity to growth cancerous cells and chemo-resistance (14). A wide range of circumstances, including DNA damage, can activate the apoptotic pathway or uncontrolled proliferation (4). Cancer cells can avoid apoptosis in a variety of ways, including inhibiting caspase activity. Caspases may also exert non-apoptotic functions, such as influencing cell proliferation, migration and the immune response.They are involved in the differentiation of several cell types, including macrophages, and may have a role in the secretion of cytokines and growth factors (28). Caspases are cysteine-dependent aspartate-specific proteases has ability to cleaving protein , the active site to cleave aspartic acid peptide bonds within proteins, after aspartic acid residues including cell death, Inflammation, Caspases are synthesized as inactive precursors termed procaspases,Caspases are present in the cytoplasm as inactive procaspases. Caspase can be classified to differentiate between initiator caspases, triggered by death receptor clustering, and caspase effector. Initiator caspases represented by caspases 2, 8, 9, and 10 while effector caspases are represented by caspases 3, 6, and 7 (21). Initiator caspases activate effector caspases in response to specific cell death signals, and effector caspases cleave various cellular proteins to trigger apoptosis (6). *Caspase 9* has a vital role in intrinsic pathway. It is initiated by release of cytochrome *c* from mitochondria in response to many cytotoxic signals such as DNA damage, cytotoxic drugs and growth factor withdrawal. Cytochrome *c* is a peripheral protein of the mitochondrial inner membrane interacts with Apaf-1, proCASP-9 and dATP results in formation of multiprotein complex apoptozome. apoptosome serves as a recruitment platform that promotes procaspase- 9 activation (29). Once a bond to apoptozome is formed, *caspase-9* is activated, which triggers other effectors such as *caspases 3* and *7* (27). Caspase 9 is mapped on chromosome 1p36.21 which is important in the mitochondrial pathway due to many factors, such as chemotherapy and radiation. *Caspase 9* is activated on the apoptosis complex to preserve catalytic status and is thought of including homo-dimerization monomeric zymogens. Miscarriage to activate *caspase 9* has intensive pathophysiological implications, contributing to cancer as well as degenerative and developmental disorders. Other proteins and small molecules included in the regulation of *caspase 9* to attained regulation of the apoptotic cycle (18, 20).

MATERIALS AND METHODS

Patients and control

Fifty patients with CML were included in this study with an age ranging between (4-75) years old divided according to gender into (19) male and (31) female, were obtained from Mosul city (Iben Sina Hospital) and Erbil city (Nanakaly Hospital), corresponding to fifty

controls with an age ranging between (20-65) years old included (27) male and (23) female and were diagnosed with CML on the basis of complete blood picture (CBC) along with bone marrow examination, and test of *BCR-ABL* gene to diagnose CML patients.

Blood collection

Samples were taken from each patients and control and the collected blood put in TRI zol 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:

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.

Three phase's 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 colorless 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 centrifuging at 12,000 for 10 minutes rpm. Total RNA precipitated into a white gel-like pellet at the bottom of the tube. After that, the supernatant is discarded.

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.

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 is measured using a spectrophotometer Nano drop (Q5000 (UV-VIS)). The purity is calculated by calculating the optical density (OD) ratio at 260/280nm.while the concentration is measured in ng/l (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*Vo/Cn

The concentration of RNA samples is normalized to the lowest concentration of sample. Where, Vn is the volume of current sample that will be diluted by TDW to generate totally 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 *CASPASE 9* genes, as well as *TEGT* as a housekeeping gene. Premier 3 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.

Gene Expression

cDNA synthesis from Mrna

The GoTaq®1-Step RT-qPCR System kit (Promega, USA) was used to assess the expression of *CASPASE 9* genes. It's a one-step RT-qPCR reagent method for quantitative RNA analysis. For 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 S stem. According to the instructions of the manufacturerd 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 liters.

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 1. Primers sequences

Primers	Sequences	Annealing Temp (C)
CASPASE9 F	5`-ACAGGGTCTGCTCTTTCT-3`	
CASPASE9 R	5`-GCATTCATCTGTCCCTCTTC-3`	60
TEGT F	5`TGCTGGATTTGCATTCCTTACA-3	
TEGT R	5`-ACGGCGCCTGGCATAGA-3`	65

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

Table2. 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

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 *CASPASE 9* gene was 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.

Primer preparation

A primer for *CASPASE 9* 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. Primers sequences for *CASPASE 9* gene was 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 show in Table 1. 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 of each Component was calculated according to the Table 4.

Table 3. Thermal cycler steps of conditions 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 template for PCR or for long-term storage at -20°C.

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 programed 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	
Annealing	60ºC	20 sec	40
Extension	72ºC	20 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**.**-Expression data of selected genes were normalized against housekeeping .The ΔΔCt method by (19) 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) 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 represented as (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=$2^{-\Delta\Delta Ct}$**

Caspase 9 protein

A quantitative detection of caspase 9 in serum of patients with CML and healthy controls were detected according to the Elisa kit based on sandwich enzyme-linked immune-sorbet assay supplied from cell biolabs, Inc.USA.

Statistical analysis

Data were statistically analyzed by utilizing Sigma plot for Windows, version 12.5 Data were appeared as mean ± standard deviation (11).

RESULTS AND DISCUSSION

Quantitative Expression of CASPASE 9

Total RNA was extracted from all samples. The concentration of total RNA ranged from 69 to 190 ng/ µl. in patients with CML While it range from 80 to191 ng/µl in healthy group. The purity of total RNA samples ranged from 1.80 to1.92 while with control group it recorded form 1.81 to1.94.

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 caspase 9 gene and housekeeping x 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 Optimal primers annealing temperature 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 temperature (ºC) was chosen by comparing the annealing temperature for forward and reverse primers (24). Quantitative expression of *caspase 9* gene and housekeeping gene *TEGT* were assessed by Real Time Polymerase Chain Reaction, in which the relative quantitation method was used. 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) respectively.

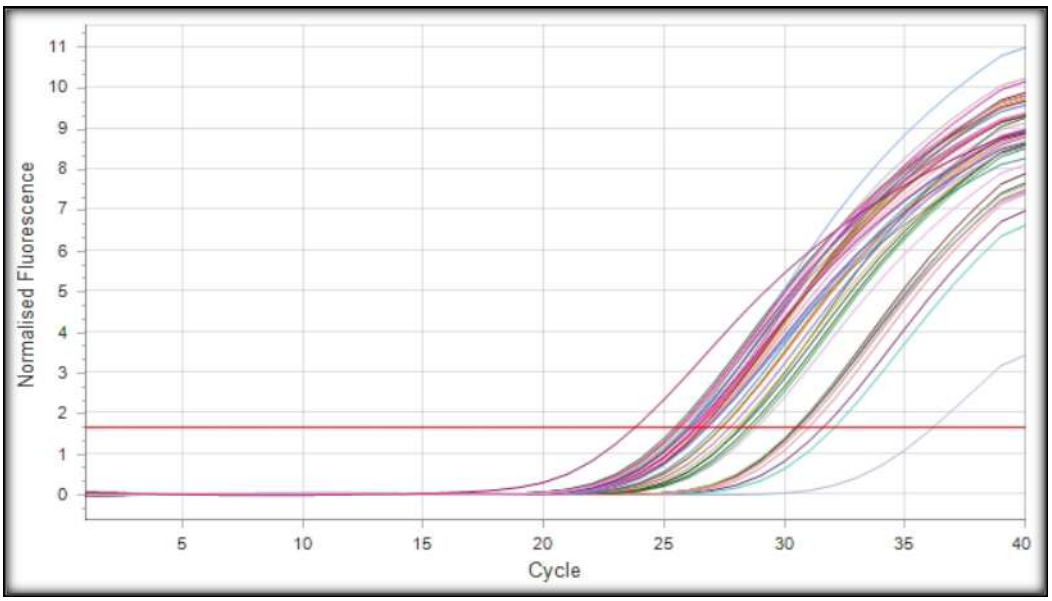

Figure 1. Amplification plots of *CASPASE 9 gene* by RT-qPCR

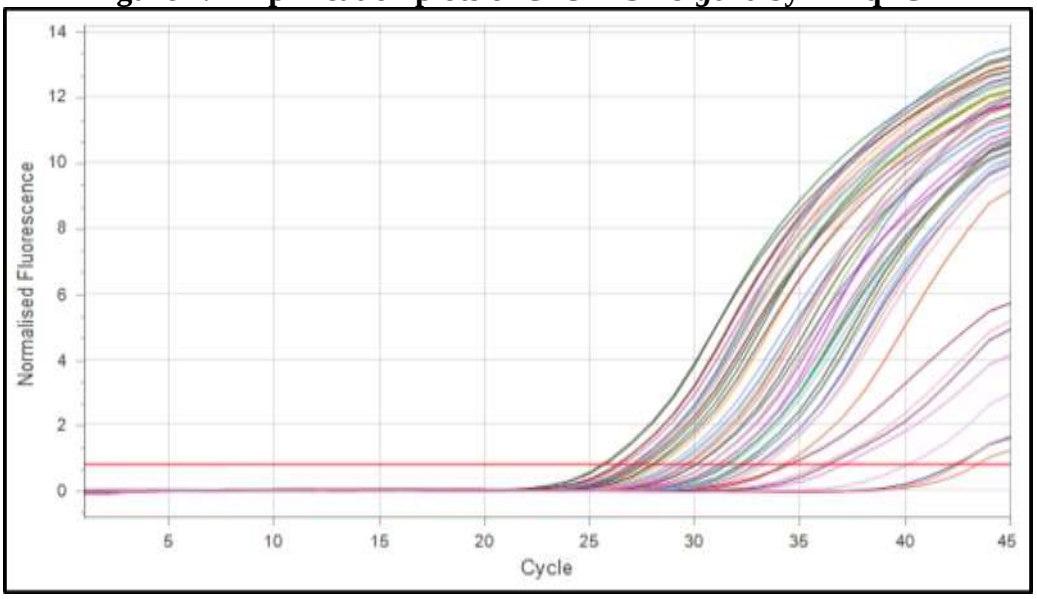

Figure 2. Amplification plots of *TEGT gene* by RT-qPCR.

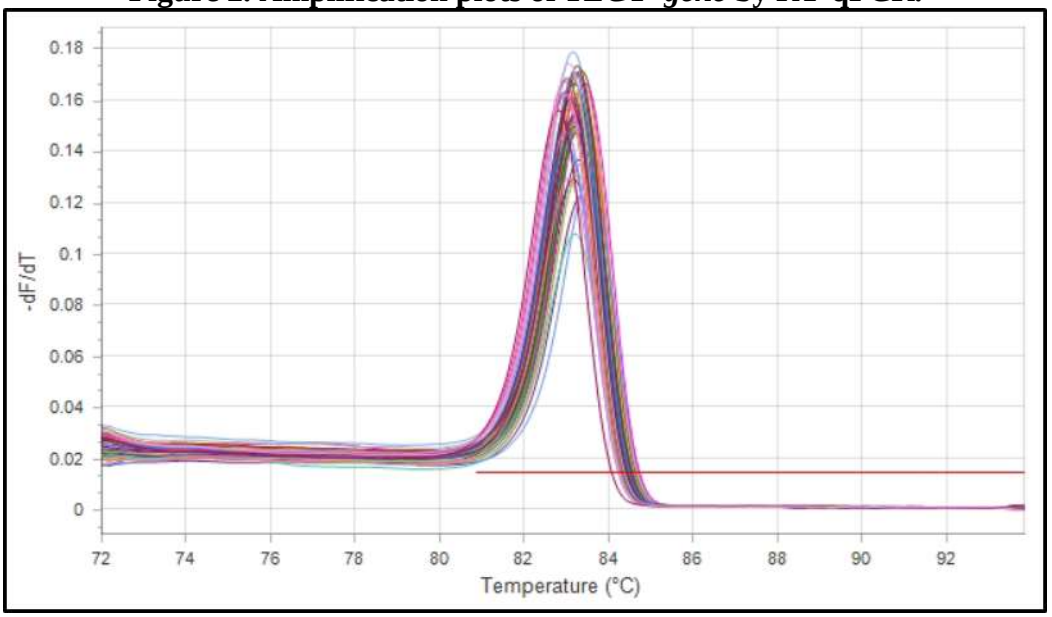

Figure 3. Melt curve of *CASPASE 9 gene* amplicons after RT-qPCR analysis showing single peaks. Threshold 0.014starting at 80.86°C

A representative melt curve *caspase 9* gene for samples analyzed by RT-PCR is shown Figure 3, in which, a single peak was observed for the amplicons. Such findings are explained 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.

Expression level of *CASPASE 9* gene in the studied groups

The ΔCt mean of *caspase 9* gene in blood samples of CML patients was (11.31) There was a significant increases $p<0.001$ in ΔCt mean of patient groups compared to the ΔCt means in control group as show in Table 6. Reveals significant differences in the expression caspase 9 mRNA and the level in CML patients reached to 16.19 as shown Table 7.

Table 6. Expression level (ΔCT) of Caspase 9 in CML patients and control groups

Gene expression Level ΔCT	Control (n=50)	Patient (n=50)	*P*
Caspase 9	5.65 ± 1.22	11.31 ± 0.89	0.001

Table 7. Mean of gene expression ($2^{-\Delta\Delta Ct}$) of CASPAS 9 mRNA in patients group

Genes	Gene expression ($2^{-\Delta\Delta ct}$) (mean± SD)	*P*
Caspase 9	16.19 ± 5.52	0.001

Table 8. Means of Caspase 9 protein in CML patients and control groups

Parameter	Control (mean± SD) (n=50)	Patients (mean± SD) (n=50)	*P*
Caspase 9 protein	6.974 ± 3.545	1.025 ± 0.212	0.001

Caspase 9 proteins have been evaluated using the enzyme-linked immunosorbent assay, Mean of Caspase 9 protein levels in CML patients showed 1.025 ± 0.212 ng/ml compared with 6.974 ± 3.545 ng/ml in control groups as shown in table 8. The results statistically revealed a significant differences $p> 0.05$ between the level of Caspase 9 protein between patients and healthy control group. These results were agreed with (15) who found CASP9 serum levels were significantly lower in the patient group with non-small cell lung cancer than in the control group. a significant correlation of serum caspase 9 levels were observed with severity of clinical state of amyotrophic lateral sclerosis and duration of the disease ($p < 0.05$) showing that caspase 9 is an important target in pathophysiology of amyotrophic lateral sclerosis (2,12). Also the inactive caspase 9 level was lower in the depigmented epidermis patient with vitiligo compared with the normally pigmented epidermis (16). Gene expression level of caspase 9 was higher in cases of CML patient when compared with healthy group, which reflect a vital role of Caspase 9 in confirming a diagnosis of CML and may explain to some extent the pathogenesis of disease (2, 8). The activity of caspase 9 is stimulated by dimerization, which leads to rapid autocatalytic cleavage, producing *caspase 9. Caspase 9* initiated apoptosis is believed to reflect the susceptibility of cancer cells to chemotherapy drugs. Deregulated apoptosis system may lead to cancer development .similar results were also obtained by (10) .the current study focus on the importance of the Caspase 9 in the development of chronic myeloid leukemia and indicate the included of Caspase 9 in the CML phases. The probable explanation for the elevated Caspase 9 gene expression level in present study is that it may have a vital role in the pathophysiologic process in this disease.In summary Caspase 9 mRNA expression were significantly unregulated in CML patients compared to controls. The data suggest the role of Caspase 9 as valuable tools for the efficiency of target treatment of chronic myeloid leukemia in Iraq.

Correlation of gender and age with CML and control groups

The results show in table 9 and 10 related with gender and age of CML patients compared with control revealed no significant differences $p>0.001$ for both gender and age of CML patients and controls. Results are in agreement with (13). The percentage of patient male was 19% while control male 27%. Moreover, patient's female exhibit 31% while control female showed 23% as shown in table 9 .Also the mean of patient's age was 40.88 while the mean of controls age was 38.82 as shown in table 10. Our result was closer to the median age in Bangladesh was 40 years old

while a median age in Europe 55 years old (14) . Genetic factors might impact the age distribution of CML patients and the high cost of medication and monitoring was another challenge for patients in low-income countries (3, 23).These results reflect that CML disease could affect both male and female at any age.

Table 9. Frequencies of control and CML patients groups depending on gender

Groups	Gender	Proportion	P
Patient (n=50)	Male	19%	0.11
Control (n=50)	Male	27%	
Patient (n=50)	Female	31%	
Control (n=50)	Female	23%	

Table 10. Means of age parameter in CML patients and control groups

Parameter	Control (mean± SD) (n=50)	Patients (mean± SD) (n=50)	*P*
Age (years)	38.82 ± 9.54	40.88 ± 15.03	0.415

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