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Evaluation of Caspase 8 Role as a Gene and Protein in Chronic Myeloid Leukemia Incidence

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Abstract
Chronic Myeloid Leukemia)CML(is a type of clonal hematopoietic stem cell disease marked by cytogenetic abnormalities induced by the growth and division of cells carrying the Philadelphia chromosome. The current research was carried out in Iraq to examine the link between Caspase 8 gene expression and Caspase 8 protein and the development of chronic myeloid leukemia (CML) in 100 samples (50 patients and 50 controls). There were differences in the expression of this gene between healthy controls and studied patients. The relationship between CML onset with age and gender was investigated in comparison to controls. The results revealed significant rises in the mean of Caspase 8 expression level (ΔCt) of patient groups in comparison to the related ΔCt means in the healthy group, as well as significant differences in gene expression folding (2-ΔΔCt) of Caspase 8 gene, which reached (2.778) in CML patients, Caspase 8 protein levels were (0.1059 ng/ml) and There were considerable variations between CML patients and control groups. On the other hand, the results for gender and age of CML patients compared to controls indicated no significant differences for genders of CML patients and controls, and significant differences P <0.0001 for age of CML patients and controls, this result suggests that CML may afflict people of different ages and affect both men and women.

Keywords: patients, age, gender, healthy, chronic myeloid

تقييم دور الكاسبيز 8 كجين وبروتين في حدوث سرطان ابيضاض الدم النيقاني المزمن

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الخلاصه
ابياض الدم النيقاني المزمن هو نوع من الامراض التي تصيب خلايا الدم الجذعيه وشخص عن الطيق الفحوصات الوراثيه الخلويه للخلايا المنقسمه التي تمحل كروموسوم فلاديلفيا. أجريت الدراسة التالية في العراق للتحقق من العلاقة بين التعبير الجيني للجين كاسبيز 8 وبروتين كاسبيز 8 وتطور سرطان ابيضاض الدم النيقاني المزمن في 100 عينه (50 مرضى و 50 اصحاء). كانت هناك اختلافات في التعبير عن هذا الجين بين المرضى ومجموعة السيطرة الاصحاء. تم التحقيق في العلاقة بين العمر والجنس مع حدوث سرطان ابيضاض الدم النيقاني المزمن بالمقارنة مع الاصحاء. كشفت النتائج عن زيادات معنوية في متوسط مستوى التعبير الجيني لمجموعات المرضى مقارنة بمتوسط المقابل في المجموعة الاصحاء ، بالإضافة إلى اختلافات

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معنوية في طي التعبير الجيني لجين الكاسبيز 8، والتي وصلت إلى (2.778) في مرضى سرطان ابيضاض الدم النيقاني المزمن ، كانت مستويات البروتين الكاسبيز 8 (0.1059 نانوغرام / مل) في مرضى سرطان ابيضاض الدم النيقاني المزمن مقارنة بمجموعة الاصحاء، مع وجود فروق معنوية. من ناحية أخرى ، أشارت النتائج الخاصة بنوع وعمر مرضى سرطان ابيضاض الدم النيقاني المزمن مقارنة بالاصحاء إلى عدم وجود فروق ذات دلالة إحصائية بين الجنسين من مرضى سرطان ابيضاض الدم النيقاني المزمن والاصحاء ، ووجود فروق ذات دلالة إحصائية P <0.0001 بالنسبة لعمر مرضى سرطان ابيضاض الدم النيقاني المزمن والضوابط ، وتشير هذه النتيجة إلى أن سرطان ابيضاض الدم النيقاني المزمن يمكن أن يؤثر على جميع المرضى. الأعمار وتحدث في كل من الذكور والإناث.

INTRODUCTION

Chronic Myeloid Leukemia (CML) is a chronic myeloproliferative illness with an initial chronic course lasting 3–5 years [1], Obesity and weight increase in adulthood are significant factors in the risk of CML [2]. There was an increased frequency of different malignancies and autoimmune disorders earlier the CML diagnosis, indicating that CML patients had an inherited predisposition to cancer and autoimmunity [3] [4]. The cause of CML is currently unclear. The main well-established risk factor in Japanese atomic bomb survivors in Hiroshima and Nagasaki is a response to high amounts of ionizing radiation [5]. This proliferation of mutant stem cells will yield some differentiated cells, resulting in an increase in overall myeloid mass [1].

CML is a type of clonal hematopoietic stem cell disease marked by cytogenetic abnormalities induced by the growth and division of cells carrying the Philadelphia chromosome [6]. In a hematopoietic cell, a reciprocal translocation between the long arms of chromosomes 9 and 22, known as the Philadelphia chromosome, occurs. The gene complex BCR-ABL1, also known as t (9; 22), was created by fusing the breakpoint cluster area (BCR) gene on chromosome 22 (22q11) with the proto-oncogene Abelson (ABL1) on chromosome 9 (9q34) (q34; q11) [7]. CML affects men more than women and can affect persons of all ages, however, it is typically a disease of the elderly [8].

In 1972, Kerr, Wyllie, and Currie coined the word "apoptosis" (from the Greek means "falling off") [9], which define as the process of cell suicide that carried out by specialized protein-cleaving enzymes called caspases [10]. It is necessary for the control of several physiological processes and for healthy development [11]. As a result, any change in the rate of apoptosis might upset this equilibrium and cause developmental flaws or clinical illnesses. For instance, insufficient apoptosis encourages the development and spread of cancer [12]. Caspases are a class of cysteine proteases with great evolutionary conservation that function as common effector molecules in diverse types of cell death [13]. Caspase-8, a cysteine protease involved in the signaling of cell death by TNF/nerve growth factor family receptors, is knocked out [14].

In humans, Together with two additional highly similar proteins, FLICE inhibitory protein (FLIP) and CASPASE 10, the CASPASE 8 gene is located on chromosome 2q33-q34 [15]. The apoptosis extrinsic pathway. The soluble TNF family ligands TNF receptor (TNFR), apoptosis-inducing ligand (TRAIL), FasL, and TNF bind to and detect their respective death receptors by forming trimers. I) FasL and TRAIL: Following ligand binding, conformational changes in DR4, DR5, and Fas lead to the development of the death-inducing signaling complex (DISC). Decoy receptor 1 (DcR1), DcR2, and DcR3 bind ligands with high affinity but do not induce apoptosis. Then, through complimentary death domains, DR4, DR5, and

Fas recruit the Fas-associated death domain (FADD) (DDs). Through their complementing death-effector domains, FADD can attract caspase 8 (DEDs). The DISC is autoproteolytically cleaved when caspase 8 is recruited to the structure, releasing two subunits that make up the active enzyme. Caspases 3, 6, and 7 are sufficiently activated by caspase 8 in type I cells to fully engage the apoptotic response. The intrinsic mechanism of apoptosis is triggered in type II cells by activated caspase 8 cleaving Bid, which prompts Bax and Bak to release components from the mitochondria, including cytochrome c. This increases the amount of active caspase 8 and activates the intrinsic process of apoptosis. II) TNF: TNF binds to TNF-R1 and activates a complex of proteins that includes receptor-interacting protein (RIP), TNFR-associated factor 2 (TRAF2), and TRADD via its DD (Complex I).

Inhibitor of nuclear factor (NF)-κB (IκB)-kinase complex can be activated by complex I, allowing NF-κB to enter the nucleus and trigger the fast transcription of genes that prevent apoptosis, such as FLICE inhibitory protein (FLIP) and cIAP1/2. After that, Complex I separates from TNF-R1 and binds to FADD and caspase 8. (Complex II). FLIP can prevent Complex II's caspase 8 from activating on its own if there is ~~an~~ enough amount of it. Otherwise, complex II results in an apoptotic reaction that is triggered by caspase 8 [15].

In addition to its well-known role as an essential apoptosis signaling protein, recent data reveals that caspase-8 also performs a number of non-apoptotic tasks. For instance, by regulating IL-2 synthesis to limit T cell multiplication, caspase-8 contributes to the maintenance of peripheral T cells' homeostasis. Additionally, the regulation of B cell, NK cell, and hematopoietic progenitor differentiation and proliferation are mediated by caspase-8 [16] [17]. This research was conducted to evaluate the role of the *Caspase 8* gene and it is an activity in the occurrence of Chronic Myeloid Leukemia in Iraqi patients.

MATERIALS AND METHODS

Patients and control

Fifty patients with CML were included in this study ranging in age from 18 to 70 years old, and were separated into two groups: (21) males and (29) females, were acquired from Baghdad city (medical city, educational laboratories) and (national center for research and treatment of hematology), corresponding to fifty controls varying ages from (11-49) years included (21) male and (29) 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

Blood was drawn from every CML patient and healthy control and then placed in a tube containing a TRIzol™ Reagent for mRNA testing.

TRIzol Extraction of Total RNA

RNA (mRNA) was extracted from samples using TRIzol™ Reagent kit by adopting the manufacturer's protocol"

RNA purity and concentration measurements

The concentration and purity of extracted mRNA were detected using Nano-drop spectrophotometer (Q5000 UV-VIS, Origin) by measuring the absorbance at 260/280 nm

Primer design

The NCBI Gen Bank database was used to obtain the cDNA sequences of the *CASPASE 8* genes, as well as β-Globin as a housekeeping gene. Premier 3 software was used to design RT-qPCR primers with a melting temperature of 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 examine the expression of CASPASE 8 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. In accordance with the manufacturer's instructions, the technique was carried out in a reaction volume of 20 µl. The total amount of RNA that needed to be reversed transcribed was 4 µl liters.

Protocol

Reverse transcription processes need to be put together in an environment free of RNase. All materials were thawed, including the RNA templates, and each solution was carefully blended. The PCR tube rack was loaded with the RT FDmix tubes.
According to Table 2, The RT FDmix tube was filled with the reaction component.

Table 1: Sequences of Primers.

Primers		Sequences 5′→3′	Annealing Temp (C)
B-Globin-F	*F*	ACACAACTGTGTTCACTAGC	65
B-Globin-R	*R*	CAACTTCATCCACGTTCACC	
CASP8-exp-F	*F*	AGAAGGGTCATCCTGGGAGA	65
CASP8-exp-R	*R*	TCAGGCTTCCTTCAAGGCTGC	

Table 2: Reverse transcription reaction components and reaction volume are used to develop cDNA from total RNA.

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

The developed cDNA was employed as a PCR template or stored at - 20°C for lengthy periods of time.

Quantitative real-time PCR (qRT–PCR):

The expression levels of the CASPASE 8 gene were assessed using reverse transcription-quantitative polymerase chain reaction (qRT-PCR). This method can accurately measure mRNA levels in a steady state. The target gene's expression was examined using a quantitative real-time qRT-PCR SYBR Green test. The primer sequences for the CASPASE 8 gene were synthesized by the current work and maintained lyophilized at -23°C by Alpha DNA Ltd. (Canada). Table 1 displays primer sequences. By increasing the endogenous

control gene β-Globin, the mRNA levels of the studied genes were brought back to normal. Table 1 also contains the primer sequences for β-globin.

Primer preparation

Macrogen Company provided a lyophilized primer for CASPASE 8 and β-Globin working solution. Lyophilized primers were diluted in nuclease-free water to achieve a stock solution concentration of 100pmol/µl. To create a functional primer solution of 10pmol/µl, 10µl of primer stock solution (stored at freezer -20 C) was mixed with 90µl of nuclease-free water.

Quantitative real-time PCR (qRT–PCR) run

The MIC-4 real-time PCR System (AUSTRALIA) was used, the quantitative real-time PCR (qRT-PCR) method was used. By monitoring the threshold cycle (Ct) while using the 2xqPCR Master Mix Kit’s components, the fold change, and gene expression levels were computed. Every action was performed twice. According to Table 4, each component's needed volume was determined.

Table 3: Thermal cycler steps for cDNA Reverse Transcription.

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

The newly created cDNA was immediately utilized as a PCR template or was kept for a long time at - 20°C.

Table 4: Quantitative real-time PCR components used in gene expression experiments.

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 routine was set up for the following optimal cycles and in accordance with the Table 5 thermal profile.

Table 5: Thermal profile of genes expression

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

The real-time cycler software was used to calculate the threshold cycle (CT) for each sample. The mean values of each sample were computed after being run in duplicate. Selected gene expression data were standardized against housekeeping. The ΔΔCt technique proposed by [18] was utilized for data analysis, and the findings were reported as folding change in

gene expression as follows: The difference between the CT values (ΔCt) for each target gene and the housekeeping gene was computed for each sample.
ΔCt (control) =CT (gene)-CT (HKG)
ΔCt (patient) =CT (gene)-CT (HKG)
The difference in ΔCt values was determined as follows (ΔΔCt) for the genes of interest:
ΔΔCt=ΔCt (patient) - ΔCt (control)
The gene expression fold-change was computed as follows:
Fold change=2-ΔΔCt

Caspase 8 protein

A quantitative detection of caspase8 in the serum of patients with CML and healthy controls was detected according to the Elisa kit based on a sandwich enzyme-linked immune-sorbet assay supplied from cell biolabs, Inc.USA.

Statistical analysis

The statistical analysis was performed using GraphPad Prism version 9.0 (GraphPad Software Inc., La jolla, CA). Student's *t-test* and one-way ANOVA (Tukey test) were used to determine whether group variance was significant or not. Chi-square was employed to test count variances. Pearson coefficient (r) was used to assess correlation. Data were expressed as mean ± SD and statistical differences were defined as * $p \leq 0.05$ *or* ** $p \leq 0.0001$ [19].

RESULTS AND DISCUSSION

Quantitative Expression of CASPASE 8

All samples had total RNA extracted. Total RNA concentrations in individuals with CML and the healthy control group varied from 10 to 20 ng/µL.

cDNA reverse transcription

The complementary DNA reverse transcription was performed on the next day following RNA extraction. Considering cDNA for caspase 8 and housekeeping genes was required, a similar primer reaction was used. The efficiency of cDNA accumulation was subsequently found using the effectiveness of qPCR. All processes were linked with ideal yield, indicating effective reverse transcription. The ideal temperature for primer annealing was found using the following equation based on the Tm of each primer specified in the manufacturer's guidelines:

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

The melting temperatures for the reverse and forward primers were also computed using the previous equation. By comparing the annealing temperatures for forward and reverse primers, the lowest temperature (ºC) was determined [20, 21]. The relative quantitation technique was utilized to analyze the quantitative expression of the caspase 8 gene and the housekeeping gene β-Globin using Real-Time Polymerase Chain Reaction.

The amount of gene expression was adjusted to that of a housekeeping gene and measured using the ΔCt value and folding ($2^{-\Delta\Delta Ct}$) methods, as illustrated in Figures (1, 2).
Figure (3) shows a typical melt curve for the caspase 8 gene for samples examined by RT-PCR, with a single peak for the amplicons. The melt curve ~~is~~ for an intercalating dye test revealed a pure, single amplicon in each sample, and the specificity of amplification was confirmed to be satisfactory.

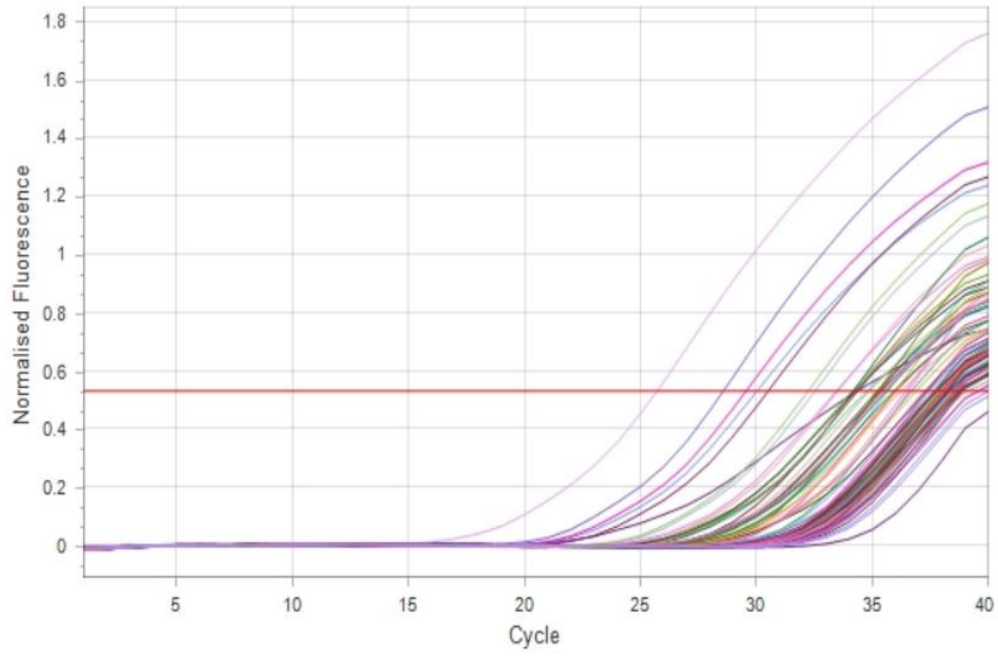

Figure 1: Amplification plots of *CASPASE 8* gene by RT-qPCR.

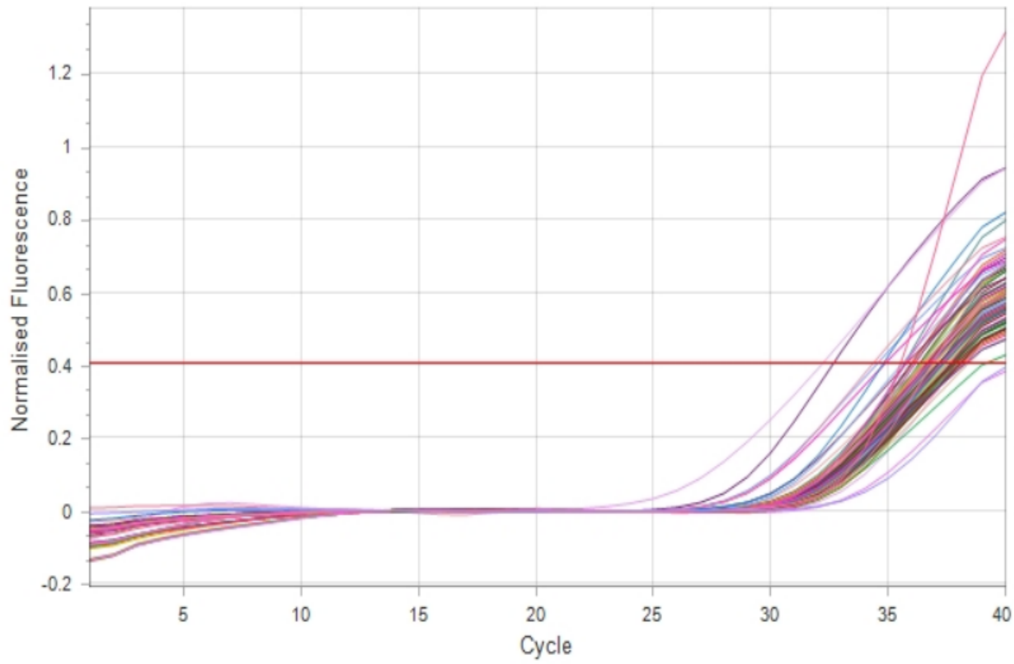

Figure 2: Amplification plots of *β-globin* gene by RT-qPCR.

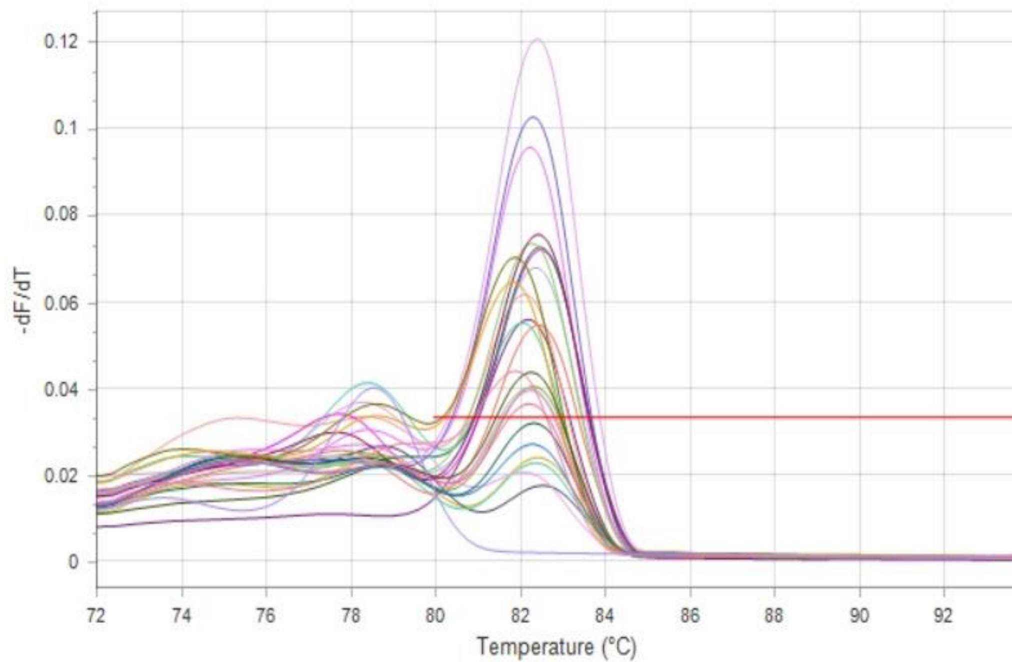

Figure 3: Melt curve of *CASPASE 8* gene amplicons after RT-qPCR analysis showing single peaks. Threshold 0.033 starting at 79.95°C.

Expression level of the *CASPASE 8* gene in the studied groups

The ΔCt mean of the caspase 8 gene in CML patients' blood samples was (0.7) There was a Significantly different $p<0.0001$ in ΔCt mean of patient groups in comparison to the ΔCt means in control group as shown in Table 6.

As indicated in Table 7, there was a significant increase in the expression of caspase 8, with the amount in CML patients reaching (2.778).

Table 6: Expression level (ΔCT) of *Caspase 8* in CML patients and control groups

	Caspase 8	
	Mean(ΔCt) Control ± SD	Mean (ΔCt) CML ± SD
	2.0 ± 0.335	0.678 ± 0.243
Sig.	**	
p Value	<0.0001	

Table 7: Mean of folding of gene expression ($2^{-\Delta\Delta Ct}$) of *CASPAS 8* in patients group.

Genes	Gene expression ($2^{-\Delta\Delta ct}$) (mean± SD)
CASPAS 8	2.778± 4.85

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

Parameter	Control (mean± SD) (n=50)	Patient (mean± SD) (n=50)	*P*-Value
Caspase 8 protein	0.264 ±0.127	0.105 ±0.0547	0.001**

The enzyme-linked immunosorbent method was used to test Caspase 8 protein levels in CML patients, which indicated 0.1059 ± 0.05472 ng/ml compared to 0.2643 ± 0.1271 ng/ml in control groups, as shown in Table 8. The results demonstrated statistically significant differences in Caspase 8 protein levels between patients and the healthy control group (*p> 0.0001*). These results were agreed with [22] who found Small cell cancers, hematologic malignancies, and brain tumors (which contain a predominantly proteoglycan-based ECM) are more likely to lack caspase-8 expression. From a clinical point of view, it may prove valuable to evaluate the expression and phosphorylation status of caspase-8 in malignancies, to examine the feasibility of employing this protein as a prognostic marker or to pharmacologically accelerate caspase-8 processing [23]. Also, the levels of caspase-8 in the epidermis of human atopic dermatitis skin were observed to be decreased [24].

Caspase 8 gene expression levels were greater in CML patients compared to healthy controls, indicating that Caspase 8 plays an important role in validating a diagnosis of CML and may explain the pathophysiology of the illness to some extent [25, 26]. Although caspase-8 expression is reduced in a subset of human cancers, it is maintained or increased in many common tumor forms, indicating a nonapoptotic, probably prometastatic activity. Caspase-8 was discovered to increase cell migration and cell-matrix adhesion when stimulated by these signals. Additional investigations verified and expanded these findings, identifying a new role for caspase-8 that might explain its developmental effects, as well as determining that activated FAS promotes invasion [23].

Gender and age correlations with CML and control groups:

The results shown in Tables 9 and 10 for gender and age of CML patients compared to controls indicated no significant differences for genders of CML patients and controls, and significant differences *P <0.0001* for age of CML patients and controls.

Results are in agreement with [27]. The percentage of male patients was 21%, while the percentage of male controls was 21%. Moreover, female patients exhibited 29% whereas the control female had 29% as shown in Table 9. In addition, the average age of the patients was 43.82, while the average age of the controls seemed to be 28.94 as seen in Table 10. Our finding was closer to the median age in Bangladesh, which was 40 years old, than the median age in Europe, which was 55 years old [28]. Genetic factors might alter the age distribution of CML patients, and the massive price of medicine and another difficulty was monitoring for patients in poor nations [29, 30]. These results suggest that CML illness can strike both men and women at any age.

Table 9: The gender distribution of control and CML patient groups

Group	Gender	Proportion	P
Patient	male	21%	no significant differences
Control	male	21%	
Patient	female	29%	
Control	female	29%	

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

Parmeter	Control (mean ± SD)	Patients (mean ± SD)	P
Age	**28.94 ± 10.30**	**43.82 ± 13.99**	**< 0.0001**

The current work focuses on the role of Caspase 8 in the progression of chronic myeloid leukemia and suggests that Caspase 8 be included in the CML stages. The most likely reason for the increased Caspase 8 gene expression level in our study is that it plays an important part in the pathophysiologic process of this illness. In conclusion, Caspase 8 mRNA expression was considerably lower in CML patients compared to controls. The findings point to Caspase 8 as a viable tool for improving the efficacy of target therapy of chronic myeloid leukemia in Iraq.

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