Al-Rafidain J Med Sci. 2025;9(1):182-188.
DOI: https://doi.org/10.54133/ajms.v9i1.2093

AJMS
Online ISSN (2789-3219)

Research Article

Evaluating *CASPASE-9* Gene Expression and Protein Level in Iraqi Patients with β-Thalassemia Major

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Received: 18 May 2025; Revised: 15 July 2025; Accepted: 3 August 2025

Abstract

Background: Caspase-9 (*CASP9)* is a cysteine-dependent, aspartate-specific protease primarily recognized for its role in apoptosis. It functions as an initiator caspase in the intrinsic (mitochondrial) pathway of apoptosis, leading to programmed cell death. ***Objectives***: To assess the gene expression of *CASP9* and its protein level in β-thalassemia major (β-TM) patients compared to healthy controls. ***Methods***: The study included 100 participants, 50 of whom had β-TM, whereas the other 50 served as controls. To assess the *CASP9* transcript levels, blood samples were collected from each participant, and RNA extraction was performed. cDNA synthesis was carried out, and real-time PCR was utilized for the analysis. ***Results***: No statistically significant difference was observed in the age distribution between the β-thalassemia major (β-TM) patients and the healthy controls ($p>0.05$), with a mean age of 15.58 and 15.24 years, respectively. The gender was comparable between groups ($p>0.05$), indicating that the groups were well-matched. The *CASP9* gene was significantly overexpressed in β-TM patients, with a fold change of 2.04 compared to 1.0 in controls. Gene expression was quantified using quantitative real-time PCR (qRT-PCR), while protein levels were measured using enzyme-linked immunosorbent assay (ELISA). A positive correlation was observed between *CASP9* mRNA expression and its corresponding protein levels ($r=0.489$, $p<0.0001$). ***Conclusions***: *CASP9* was overexpressed in β-TM patients and was positively correlated with its protein level, indicating that transcriptional upregulation is associated with increased protein expression in β-TM patients.

Keywords: Beta thalassemia major, *CASP9* gene, Real Time PCR.

تقييم التعبير الجيني *CASPASE-9* ومستوى البروتين لدى المرضى العراقيين المصابين بالثلاسيميا β الكبرى

الخلاصة

الخلفية: *(CASP9)* Caspase-9 هو بروتياز يعتمد على السيستين وخاص بالأسبارتات معترف به في المقام الأول لدوره في موت الخلايا المبرمج. إنه يعمل كبادئ كاسباز في المسار الجوهري (الميتوكوندريا) لموت الخلايا المبرمج، مما يؤدي إلى موت الخلايا المبرمج. **الأهداف:** تقييم التعبير الجيني ل *CASP9* ومستوى البروتين في مرضى β الثلاسيميا الكبرى (β-TM) مقارنة بالضوابط الأصحاء. **الطرائق:** تضمنت الدراسة 100 مشارك، 50 منهم لديهم β-TM، في حين أن ال 50 الآخرين كانوا بمثابة ضوابط أصحاء. لتقييم مستويات نسخ *CASP9*، تم جمع عينات الدم من كل مشارك واستخراج الحمض النووي الريبي. تم إجراء تخليق cDNA، وتم استخدام تفاعل البوليميراز المتسلسل في الوقت الفعلي للتحليل. **النتائج:** لم يلاحظ فرق يعتد به إحصائيا في التوزيع العمري بين مرضى الثلاسيميا الكبرى (β-TM) والأشخاص الأصحاء ($p>0.05$)، بمتوسط عمر 15.58 و 15.24 سنة على التوالي. وكان نوع الجنس قابلا للمقارنة بين المجموعات ($p>0.05$)، مما يدل على أن المجموعات كانت متطابقة بشكل جيد. تم التعبير عن جين *CASP9* بشكل مفرط في مرضى β-TM، مع تغيير أضعاف قدره 2.04 مقارنة ب 1.0 في الضوابط. تم قياس التعبير الجيني باستخدام تفاعل البوليميراز المتسلسل الكمي في الوقت الفعلي (qRT-PCR)، بينما تم قياس مستويات البروتين باستخدام مقايسة الممتز المناعي المرتبط بالإنزيم (ELISA). لوحظ ارتباط إيجابي بين تعبير *CASP9* mRNA ومستويات البروتين المقابلة له ($r = 0.489$ ، ص <0.0001). **الاستنتاجات:** لوحظ زيادة مفرطة في التعبير *عن CASP9* في مرضى β-TM وكان مرتبطا بشكل إيجابي بمستوى البروتين، مما يشير إلى أن تنظيم النسخ يرتبط بزيادة التعبير عن البروتين في مرضى β-TM.

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Article citation: Abbas RM, Al-Saffar JMJ. Evaluating *CASPASE-9* Gene Expression and Protein Level in Iraqi Patients with β-Thalassemia Major. *Al-Rafidain J Med Sci*. 2025;9(1):182-188. doi: https://doi.org/10.54133/ajms.v9i1.2093

INTRODUCTION

One of the prevalent monogenic hereditary disorders leading to anemia worldwide was thalassemia. It is primarily categorized α-thalassemia and β-thalassemia. In β-thalassemia, anemia arises from both ineffective erythropoiesis and a decrease in the lifespan of circulating red blood cells. This leads to the accumulation of toxic, unmatched α-globin chains, which are believed to induce apoptosis of erythroid precursor cells in the bone marrow [1,2]. One of the major pathogenic mechanisms leading to anemia in different acquired and inherited disorders is ineffective erythropoiesis, which is characterized by apoptosis of

matured nucleated erythroid cells or premature death of erythroid precursors in bone marrow. Furthermore, it also has a critical role in the pathophysiology of thalassemia patients [3]. Apoptosis, commonly known as programmed cell death, was first identified in the 1970s as a distinct and tightly regulated biological process. It plays a crucial role in preserving tissue homeostasis in adults by maintaining a balance with mitosis, thereby regulating cell populations. Apoptosis is also essential during embryonic development and contributes to various biological processes, including immune system regulation, elimination of damaged or abnormal cells, and response to cellular stress. [4]. Apoptosis is regulated by several signaling pathways, such as the intrinsic (mitochondrial) and extrinsic (death receptor) pathways. Initiator caspases, such as Caspase-8 and Caspase-9, activate downstream executioner caspases, including Caspase-3, -6, and -7**,** which have critical roles in apoptotic signaling cascades. The extrinsic apoptotic pathway is triggered by the binding of extracellular ligands—such as tumor necrosis factor (TNF)—to their corresponding death receptors on the cell surface. This interaction leads to the formation of the death-inducing signaling complex (DISC), which in turn activates caspase-8, initiating a cascade that activates downstream executioner caspases [5]. Caspase-9, a cysteine-aspartic protease recognized for its function as an activator of intrinsic apoptosis, governs normal cell death and pathological tissue degeneration. Additionally, it possesses non-apoptotic functions, including the regulation of cellular differentiation and maturation, innate immunity, mitochondrial homeostasis, and autophagy [6]. Caspase-9 consists of three domains: an N-terminal pro-domain, a large subunit, and a small subunit [7]. Caspase-9 can trigger the intrinsic pathway of apoptosis through multiple mechanisms, whereby signals induce selective permeability of the mitochondrial inner or outer membrane, resulting in the release of proteins from the intermembrane gap into the cytosol. The Bcl-2 protein family participates in the development of macropores in the outer mitochondrial membrane [8]. Cytochrome-c, after release from the mitochondrial intermembrane space into the cytosol, leads to the formation of a protein complex known as the apoptosome. This complex facilitates the cleavage of procaspase-9 into its active form, caspase-9. Activated caspase-9 subsequently cleaves and activates procaspase-3, producing effector caspase-3. This leads to the systematic dismantling of the cell during apoptosis [9]. The current clinical treatment of β-thalassemia primarily involves iron chelation therapy and regular blood transfusions. While allogeneic hematopoietic stem cell (HSC) transplantation acts as a critical cure, its application is significantly limited by the availability of HLA-compatible donors [10]. CRISPR-Cas9 technology represents a powerful gene-editing technology that is used for treating diseases such as β-thalassemia. The CRISPR-Cas9 system comprises two essential components: the Cas9 enzyme, which functions as 'molecular scissors' to cleave both strands of DNA at a precise genomic location, thereby facilitating the addition or removal of DNA segments, and a piece of RNA known as guide RNA (gRNA). This comprises a short, pre-designed RNA sequence (about 20 bases in length) situated within a longer RNA scaffold. The scaffold component attaches to DNA, while the predetermined sequence directs Cas9 to the appropriate region of the genome. This ensures that the Cas9 enzyme cleaves at the correct locus in the DNA. This method has some major advantages, including high genome editing efficiency and low cytotoxicity [11]. This study aims to evaluate the gene expression of Caspase9 in patients with β-thalassemia using quantitative real-time PCR (qRT-PCR) and to assess its corresponding protein levels through enzyme-linked immunosorbent assay (ELISA). Additionally, the study investigates the role of Caspase9 in disease pathogenesis and explores its potential as a therapeutic target to improve understanding and management of β-thalassemia.

METHODS

Study design and sample selection

In this case-control study, 100 Iraqi people were recruited and categorized into two distinct groups: patients and controls. The patient group consisted of 50 individuals (27 females and 23 males) with an average age range of 15.58 ± 6.83 years. Participants were recruited from the Genetic Hematology Center at Ibn Al-Baladi Hospital in Baghdad, Iraq, and were diagnosed at the specified center based on electrophoresis of hemoglobin, complete blood count (CBC), ferritin assays, and clinical manifestations associated with the condition evaluated by the facility's physicians and consultants. According to the age of the patient at diagnosis, patients were assigned to one of two groups: from 2 to 15 years and from 16 to 29 years. The control group comprised 50 healthy individuals (25 females and 25 males) with a mean age of 15.24±7.06 years. These participants were obtained from the National Blood Transfusion Center. They were selected based on age, had no history or clinical evidence of beta-thalassemia or any other disease, and were matched in terms of age and sex with the patients in the study.

Sample collection

The samples used in this study were collected from the Genetic Hematology Center, Ibn Al-Baladi Hospital, Baghdad, Iraq, between November 2023 and February 2024. Every participant in this study had a venipuncture performed to obtain blood samples, with a volume of 5 ml extracted using disposable syringes. Total RNA was

extracted from EDTA-treated whole blood samples using a commercially available RNA extraction kit (TransGen Biotech, China), following the manufacturer's instructions. The concentration and purity of the extracted RNA were determined using a NanoDrop spectrophotometer by measuring absorbance at 260 and 280 nm. RNA samples with an A260/A280 ratio between 1.8 and 2.0 were considered suitable for downstream analysis. Complementary DNA (cDNA) was synthesized from 1 µg of total RNA using the Reverse Transcriptase Kit (Add Bio, Korea), according to the manufacturer's protocol. The resulting cDNA was stored at –20°C until used for quantitative real-time PCR. The serum produced was used for assessing caspase-9 protein levels with an ELISA kit (Sun Long Biotech, China).

Estimation of Caspase9 protein level

Serum caspase-9 levels were quantified using a commercially available human caspase-9 ELISA kit (SL2446Hu, Sun Long Biotech, China), according to the manufacturer's instructions. Serum samples were diluted 1:5 with sample diluent prior to assay. A total volume of 100 µL of each diluted sample, standard, and blank was added to the wells of the microtiter plate pre-coated with an anti-human Caspase9 antibody. The assay included a standard curve generated from serial dilutions of known caspase-9 concentrations provided in the kit. All samples, standards, and blanks were measured in duplicate to ensure accuracy and reproducibility. After incubation and washing steps, TMB substrate was added, followed by a stop solution. The absorbance was measured at 450 nm using a microplate reader. The concentration of Caspase9 in each sample was calculated by comparing the absorbance to the standard curve using a four-parameter logistic (4-PL) curve-fitting method. Results were expressed in ng/mL.

Purification of RNA

Total RNA was extracted from EDTA-treated whole blood samples using the TRIzol reagent (Transgene/China). Initially, 300 µl of whole blood was transferred into a tube containing 600 µl of TRIzol reagent, mixed gently, and kept at -20°C until its extraction by using an extraction kit (TransGen Biotech, China). The cells in the sample were lysed by vortexing multiple times, followed by a 5-minute incubation at RT. To extract the RNA-containing aqueous phase, add 0.2 mL of chloroform. Subsequently, 0.5 mL of absolute ethanol was introduced to precipitate the RNA, resulting in a white, gel-like pellet. The washing of the RNA was carried out by adding 0.4 mL of 70% ethanol. Lastly, the pellet was rehydrated in 25 µL of RNase-free water and incubated at RT for one minute. The purity and concentration of the extracted RNA were measured using a Quintus Fluorometer (Promega, USA).

Reverse transcription

The process involved in this study was the conversion of all RNA into complementary DNA (cDNA) using the Add Script Reverse Transcriptase Kit (Add Bio, Korea). The manufacturer's instructions were followed, and the reaction took place in a volume of 20 µl. All RNA types were transformed into cDNA during this procedure.

Quantitative real-time PCR

Following cDNA synthesis, quantitative real-time PCR (qRT-PCR) was performed using gene-specific primers to evaluate the expression of CASP9. The ACTB gene (β-actin) was used as an endogenous reference for normalization. Primer sequences used for *CASP9* and ACTB are CASP9 forward primer 5'-AGGCCCCATATGATCGAGGA-3' *and* CASP9 reverse primer 3'-TCGACAACTTTGCTGCTTGC-5', and for the reference gene, *β-actin* forward primer 5'-AGAGCCTCGCCTTTGCC-3' and *β-actin* reverse primer 3'-GGGGTACTTCAGGGTGAGGA-5' These primers were carefully designed, and their specificity was verified using the Primer BLAST tool on the National Center for Biotechnology Information (NCBI) website. Furthermore, to optimize the PCR process, annealing temperatures were systematically optimized. This was achieved using a gradient thermal cycler (Eppendorf, Germany) with a range of temperatures. A specific protocol was employed to amplify cDNA samples through qPCR reactions. To achieve a final volume of 20 µL, the following components were added: 10 µL of 2×TransTaq® HiFi PCR SuperMix II (Promega, USA), 1 µL (0.1 µM) from each primer, 5 µL of cDNA sample, and nuclease-free water. The RT-PCR (Qiagen, Germany) was used to amplify the qPCR reactions. The qPCR program included one denaturing cycle at 94°C for 1 minute during pre-incubation, followed by forty amplification cycles. Each amplification cycle included denaturation at 94°C for 30 seconds and annealing at 60°C for 30 seconds. Finally, one final cycle was added for the melt range of 55-99°C.

Data analysis of RT-qPCR

The expression of the *Caspase9* gene was assessed using the double delta Ct (threshold cycle) (ΔΔCT) analysis (folding change), with the B-ACTIN serving as the housekeeping reference gene [11]. The following were the calculations:

ΔCT = CT of target gene – CT of reference gene ..... Eq. 1

$\Delta\Delta CT$ = ΔCT of each sample – average control ΔCT Eq. 2

Folding change = 2-ΔΔCt …… Eq. 3

Ethical approval

The College of Science Ethics Committee approves the research proposal to be conducted in the presented form. None of the investigators and co-investigators participating in this study took part in the decision-making and voting procedure for this study. The College of Science Ethics Committee expects to be informed about the study's progress, any serious adverse events occurring in the course of the study, any revision in the protocol and patient information/informed consent, and asks to be provided with a copy of the final report. This ethics committee works under the College of Science guidelines on biomedical research. The study protocol and the subject information and consent form underwent evaluation and received approval from the institutional ethics committee (CSEC/0525/0061, date: May 11, 2025).

Statistical analysis

Scale indicators were expressed like Mean±SD. The student's t-test is utilized to show statistical differences between two groups, and the F-test is used among more than two groups. Anthropometric features (gender, age, and BMI) are seen as frequencies with percentages, and variations among these percentages were screened by the Pearson chi-square test. Receiver operating characteristic (ROC) curve has been utilized to show area under the curve (AUC), cutoff, specificity, and sensitivity of all scale indicators. The Pearson correlation coefficient was used to detect the type and strength of the relationship between indicators. *P≤0.05* was depended on to select significant differences. Data from our investigation were processed by SPSS v. 23.0 and GraphPad Prism v. 6 statistical software programs.

RESULTS

The present study involved 100 subjects classified into 2 groups: β-TM patients and healthy controls. The age distribution between the β-TM patients and the healthy controls seems very similar, ranging from 2 to 29 years (15.58 ± 6.83). There were 27 females (54%) and 23 males (46%), whereas the age of the controls ranged from 2 to 29 years (15.24 ± 7.06). There were also 25 females (50%) and 25 males (50%). The study found no significant differences in the age and gender distribution between the two groups, as detailed in Table 1.

Table 1: Distribution of patients with β-TM and control group according to sex and age

		Patients	Controls	Total	*p*-value
Sex	Males	23(46)	25(50)	48(48)	>0.05
	Females	27(54)	25(50)	52(52)	
Age (year)		15.58±6.83	15.24±7.06	100	>0.05

This was done to ensure an objective and scientific comparison between the β-TM and control group patients. Based on this research, the level of *Caspase9* expression among individuals suffering from β-TM was higher than in healthy individuals. The study observed an increase in *CASP9* gene expression in a blood sample obtained from individuals diagnosed with β-TM, which was statistically significant ($p< 0.01$). The fold change in *CASP9* expression in β-TM patients (2.04) was considerably higher compared to the controls (1.0), as depicted in Figure 1.

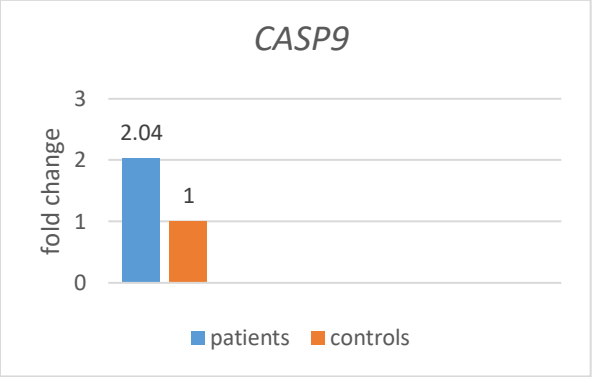

Figure 1: The fold change difference of *CASP9* between β-TM patients and control groups.

To evaluate the sensitivity and specificity of *Caspase9*, the ROC curve was employed. ROC curve results showed 61% sensitivity and highest specificity of 100% at cutoff 1.00. Furthermore, the calculation of the area under the curve (AUC) yielded a *p*-value of 0.48, as in Figure 2.

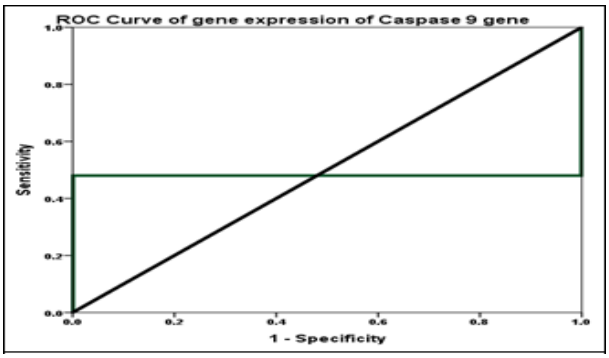

Figure 2: Receiver Operating Characteristic curve of the *CASP9*.

According to this research, there were no significant differences in folding change of *Caspase 9* with age groups of patients (Table 2).

Table 2: Comparative folding change of *CASP9* genes between age groups of β-TM

Age groups (year)		n	Mean±SD	*p*-value
CASP9	2-15	28	2.81±0.7	>0.05
	16-29	22	1.06±0.5	

Additionally, the levels of *CASP9* protein were found to have a significant increase in β-TM (823.89±281.38

ng/ml) compared to healthy controls (425.64 ± 189.51 ng/ml), according to the data shown in Table 3.

Table 3: Comparative of CASP-9 concentrations among groups of study

Groups		n	Mean±SD	*p*-value
CASP-9 *(ng/ml)*	Patients	50	823.89±281.38	<0.05
	Controls	50	425.64±189.51	

Additionally, the level of *CASP9* expression was found to have a significant positive correlation with its protein level (r = 0.489 and $p< 0.0001$), according to the data shown in Table 4.

Table 4: Correlation analysis of the *CASP 9 expression* levels and its protein level in the β-TM patients

		CASP-9 *(protein)*
***CASP9* (gene)**	r	0.489
	p-value	<0.0001

r: Pearson's correlation coefficient.

DISCUSSION

Caspase-9, also known as an apoptotic initiator enzyme, is an aspartate-specific cysteine protease that plays a central role in the intrinsic (mitochondrial) pathway of apoptosis. Upon activation, caspase-9 forms a complex with Apaf-1 and cytochrome c, leading to the activation of downstream effector caspases, such as caspase-3 and caspase-7, which execute the apoptotic process. However, recent research has revealed another function of Caspase-9 that can lead to non-apoptotic roles; these include autophagy, regulation of cell homeostasis, differentiation, and activation of NF-KB pro-survival pathways [12]. This study categorized the outcomes into two groups: patients and controls. Interestingly, no significant age difference was observed between these two cohorts, as indicated in Table 1. The recent study's findings demonstrated that compared with females (54%), males (46%) are significantly more likely to have β-TM than men. It was sure that the female immune system was affected by many physiological and biological parameters like periods, pregnancy, breastfeeding, and others; all of these changed the hormone status and subsequently affected the immune system. Hemolytic anemia is the primary pathological hallmark of β-thalassemia, arising from two main mechanisms: the premature destruction of erythroid precursor cells in the bone marrow and the reduced lifespan of mature erythrocytes in the circulation. The anemia characteristic of β-thalassemia results from ineffective erythropoiesis (IE), a process in which erythroid progenitors fail to mature properly and undergo apoptosis before reaching full differentiation [13] that is characterized by 1) expedited erythroid development, 2) maturation arrest at the polychromatophilic stage, and 3) demise of erythroid precursors. Atypical erythroid cell maturation and differentiation are characteristic of IE, which is not the primary cause of β-thalassemia, although it perpetuates a detrimental condition in individuals with β-thalassemia [14]. In thalassemia, the abnormal red blood cells and ineffective erythropoiesis can impair the body's ability to clear immune complexes, which are involved in the development of IgA nephropathy (IgAN), a kidney disease where IgA antibodies deposit in the kidneys, causing inflammation and potential damage [15]. In addition to ineffective erythropoiesis, hemolytic anemia may also result from the production of autoantibodies by the immune system, which mistakenly targets the body's own red blood cells. This autoimmune response leads to autoimmune hemolytic anemia (AIHA), further exacerbating the severity of anemia in thalassemia patients [16]. In this investigation, it can be observed from the data presented in Figure 1 that there is a significant rise in the expression of caspase-9 among individuals with beta thalassemia major compared to those without the disease. This discovery is consistent with a study by Walter *et al*. (2013) [17], where they identified that patients with beta thalassemia exhibit elevated Caspase9 activity, which correlates with increased levels of pro-apoptotic proteins like Bax and a higher Bax/Bcl-2 ratio. This indicates that caspase-9 plays a key role in the intrinsic (mitochondrial) apoptotic pathway, which is crucial for regulating programmed cell death in various tissues, including hematopoietic cells. In β-thalassemia, the decrease or absence of β-globin production in erythroblasts causes imbalance in the amount of α-globin and β-globin over the development of erythroid cells. Additionally, excess α-protein is accumulating on the cell membranes, causing damage to the erythroid precursor cells that lead to developed anemia [18]. This discovery is consistent with another study in Turkey by Elsahookie *et al.* [19], which revealed elevated levels of Caspase 9 gene expression in chronic myeloid leukemia (CML) patients compared to a healthy cohort, underscoring the critical role of Caspase 9 in diagnosing CML and potentially elucidating aspects of the disease's pathogenesis. The receiver operating characteristic (ROC) curve shown in Figure 2 illustrates that the *Caspase9* gene expression has an area under the curve (AUC) of 0.48, suggesting that it is not diagnostically useful in the current setting and might need to be reassessed with stronger biomarkers in patients with β-TM. Ficarra *et al.* [20] observed a significant increase in Caspase-3 expression in patients with β-thalassemia compared to healthy individuals. This elevated caspase-3 activity correlates with the excessive apoptosis of erythroid precursor cells, a hallmark of ineffective erythropoiesis. In β-thalassemia major, the bone marrow harbors approximately 6 times more erythroid precursors than normal. However, quantitative analyses revealed that the rate of apoptotic cell death in these patients was 4 times higher than in healthy controls, contributing to the failure of proper erythroid maturation and the persistence of anemia [21]. In contrast, Urbani *et al.* [22] reported low Caspase-8, Caspase-9, and Caspase-3

activity in human thalassemic erythroblasts. These findings suggest that upregulation of autophagy, a cellular process that acts as a protective mechanism, suppresses apoptosis and promotes cell survival. However, in other contexts, autophagy can act as an alternative form of programmed cell death, depending on the balance between cellular stress and adaptive capacity. In addition, this study found a positive correlation between *CASP9* gene expression and its corresponding protein level. This correlation suggests that transcriptional regulation of *Caspase9* contributes to increased protein expression, thereby enhancing apoptotic activity. This supports the biological role of the genes in disease pathophysiology, since caspase-9 is a key initiator of programmed cell death, the results presented in Table 4. Previous research has introduced a novel therapeutic strategy for β-thalassemia based on genome editing of the α-globin locus in human hematopoietic stem and progenitor cells (HSPCs) using the CRISPR-Cas9 system. This approach combines two key interventions: 1) downregulation of α-globin expression by deleting the *HBA2* gene to mimic an α-thalassemia trait, thereby reducing the α/β-globin imbalance; and 2) restoration of β-globin expression by targeted integration of a β-globin transgene downstream of the *HBA2* promoter [23].

Conclusion

Research on the *CASP9* gene is expected to have a significant impact on our understanding of the pathophysiology of β-thalassemia disease. In β-TM patients, *CASP9* was highly expressed, linked to enhanced apoptosis and contributing to the disease's ineffective erythropoiesis. Its expression level may serve as a potential indicator of apoptotic activity and disease progression. However, modulating CASP9 expression or blocking its activity could be a potential therapeutic strategy to improve erythropoiesis in β-TM. Through genome editing technologies, CRISPR-Cas9 technology offers a novel and effective approach to treating these conditions.

ACKNOWLEDGMENTS

The authors thank the participants and Genetic Hematology Centre at Ibn Al-Baladi Teaching Hospital for their invaluable assistance in conducting the study.

Conflict of interests

The authors declared no conflict of interest.

Funding source

The authors did not receive any source of funds.

Data sharing statement

Supplementary data can be shared with the corresponding author upon reasonable request.

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