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Genetic Expression of FAS and miRNA34a-5P with β-Thalassemia Major in Iraqi Patients: A Case-Control Study

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ABSTRACT

Beta-thalassemia major (β-TM) is an inherited hematological disorder caused by mutations in the hemoglobin beta (HBB) gene, which encodes the β-globin chain; nevertheless, the micro ribonucleic acid 34a (miRNA34a-5P) gene is a key component of HBB gene defects. This miRNA34a-5P regulates fundamental aspects of erythroid cell survival, maturation and oxidative stress. Oxidative stress, excessive iron, and unpaired α-globin chains from decreased β-globin production may induce fetal alcohol syndrome (FAS) gene expression and thus increase the rate of apoptosis of erythroblasts. Thus, the gene expression of FAS and miRNA34a-5P was amplified and quantitatively by RT-qPCR in β-TM patients from Diwaniyah, Iraq and show relative to a housekeeping gene (GAPDH). When comparing the β-TM case (G2) with the control (G1), expression levels of both FAS and the microRNA34a were significantly different ($P - value < 0.001$). The second replication study confirms these above findings that the overexpression and amplification associated, respectively, of RT-PCR for FAS and miRNA34a-5P, as well that a correlation have been noted between the genes FAS and miR 34a with their appropriate diagnostic indication for the studied disease.

1. INTRODUCTION

Beta-thalassemia major (β-TM) is a common and severe inherited hematological disease characterized by reduction in synthesized hemoglobin (Hbb) caused by mutations in the human Hbb gene cluster on chromosome 11 which translates into the iron-containing protein in erythrocytes that is responsible for transporting oxygen to the body[1]. Multiple organ complications are common in β-thalassemia major but are rarely present in the same way. The heart can become dysfunctional, which, depending on the time course, can present as dilated cardiomyopathy or intermittent arrhythmias, and hepatic damage can occur, leading to fibrosis that can progress to cirrhosis [2]. The other endocrine diseases with inadequate work of several glands parathyroid, thyroid, pituitary, and adrenal glands not working sufficient for physiologic need, diabetes mellitus and hypogonadism[3]. Gene expression is a process that transforms information from a gene into a functional gene product. This process produces proteins or non-coding RNA, which in turn affects a phenotype [4]. Gene expression, which ultimately plays a major role in establishing a phenotype, is the process by which a gene's information is utilized to synthesize a functional gene product, such as a protein or non-coding RNA. The transcription of the FAS gene to mRNA and subsequent translation of the mRNA to the FAS protein constitute the expression of FAS genes [5]. Reduced β-globin synthesis can potentially combine to increase the expression of FAS and increase erythroblast death through oxidative stress, iron deposition and the aggregation of unpaired α-globin chains [6]. Excessive unpaired α-globin chains lead to oxidative stress, resulting in free heme release and promoting the production of reactive oxygen species (ROS), the main contributor to β-thalassemia pathogenicity [7]. By targeting the 3' UTR of multiple mRNAs, miRNA34a-5P represses their translation and increases the death of erythroid precursors, thus preventing them from maturing to functional adult red blood cells [8]. In addition, the oxidative stress burden of β-thalassemia is aggravated by the aberration of miRNA34a-5P [9]. The disorder is associated with iron overload due to ineffective

erythropoiesis and iron pillaging from excessive hemolysis [10]. This study aims to estimate levels of parameters (FBS, HbA1c, Ferritin, TC, TG, Lipase, ALT, ALP, AST, AST/ALT ratio, Albumin, total bilirubin, globulin, albumin/globulin ratio, Total Protein, T4, TSH, Creatinine, blood urea, HGB and RBCs) and genetic expression of FAS and miRNA34a-5P in the Beta-Thalassemia major Patients compared to the control.

2. EXPERIMENTAL SECTION

Study Population

The study was a case-control study, population consisted of 180 persons, comprised 90 β-TM (G2), and 90 ethnically matched healthy controls of Iraq ethnicity (G1). The date of collecting samples 27/1/2024 and its completion date 18/7/2024. The cases included consecutive non-smoking patients, their ages (15-30) years of age who participated the Thalassemia center and Diwaniyah Teaching Hospital in Diwaniyah, Iraq.

Clinical Profile Test

A 5 ml venous blood sample was taken from each participant in the research groups (β-TM (G2) and controls (G1). The serum was then separated using a 4000-x g centrifuge set at room temperature for around 10 minutes. Both the entire blood and the serum were stored frozen at -20 °C until they were used for different assays. The first part of the study was special tests for the following biomedical parameters (HGB, and RBCs) were performed directly by the Hemolyze® 3 NG (STERILAB, UK) device by taken 1 ml of blood to estimate it. 3 ml of serum used to estimate (FBS, HbA1c, Ferritin, TC, TG, ALT, ALP, AST, albumin, total bilirubin, globulin, Total Protein, T4, TSH, Creatinine, and blood urea) were performed directly by the Beckman Coulter Au 480 (Beckman Coulter, USA) device.

Genetic Expression

RNA Extraction

A blood sample was collected in 5 mL amounts from each participant in the two study groups (G1 and G2) for further analysis of their RNA. Genomic RNA was extracted from blood using the RNA Extraction Kit (ADD Bio/Korea) at 20 µg/ml or less. One whole blood sample of two hundred microliters was lysed, in a 1.5 ml micro centrifuge tube, using six hundred microliters of Triazole and letting the sample incubate for five minutes at room temperature Two hundred microliters of chloroform were added to this mixture, which was then centrifuged at 13,000 rpm for 10 min, and the resulting supernatant was placed in a new tube. Isopropanol (600 µl) was added to the sample, vortexed, and incubated at 4 °C for 10 min. Centrifugation was performed for 10 minutes at 13,000 rpm to wash the precipitated RNA with 600 µl of 70% ethanol. The tubes were then centrifuged (10 min, 13,000 rpm), and the wash was added and allowed to cool (50 µl elution buffer). After this, the supernatant was discarded.

Estimation of RNA Concentration

After RNA extraction, absorbance at 260 and 280 nm was measured using a Nanodrop instrument to determine the concentration and purity of RNA. 1X TE buffer (pH 7.5) was made from nuclease-free water and 20X TE buffer. In order to make a working solution, 3,990 µl of 1X TE buffer was used to dilute 10 µl of Quant Fluor® Dye until the final concentration was 1:400. This ensured that the pigment was dispersed uniformly. 200 µl of Quant Fluor® RNA Dye was added to a 0.5 ml PCR tube to create the Quant Fluor®, ONE RNA System blank sample. Following the manufacturer's instructions, the nucleic acid standard (RNA) was added to a 0.5 ml PCR tube (2 µl of standard to 200 µl of working solution). Determine the standard metrics based on the results of the Quants Fluorometer. Next, evaluate the RNA samples using Quants protocols. 10 to 65 ng/µl for concentration and 1.7 to 1.9 for purity.

cDNA synthesis

The Promega (USA) Quants TM Fluorometer is used for the test. A cDNA synthesis kit (ADD Bio/Korea) may be used to convert whole blood RNA into cDNA. Each RNA sample is aliquoted into a PCR tube using random hexamer oligonucleotides, cDNA, dNTPs, and 2X reverse transcriptase. able to use a glass cDNA synthesis kit (ADD Bio/Korea) to transform chromosomal material (cRNA) from whole blood into CDNA. Housekeeping: GAPDH: $5' - GAAGGTGAAGGTCGGAGTC - 3'$ (Forward), $5' - GAAGATGGTGATGGGATTTC - 3'$ (Reverse), FAS gene: 5'-TGAAGGACATGGCTTAGAAGTG-3' (Forward), $5' - GGTGCAAGGGTCACAGTGTT - 3'$ (Reverse), miRNA34a-5P: $5' - AGGGT\ GCA\ GTG\ TCT\ TAG\ C - 3'$ (Forward) and $5' - GAG\ CCG\ AGG\ T$ (Reverse). Bio-Rad (USA) was subjected to thermal conditions.

RT-QPCR AMPLIFICATION

Using an RNA Isolation kit (ADD Bio/Korea) at 20 µg/ml or less, genomic RNA was extracted from whole blood. After RNA extraction, a Nanodrop instrument was used to evaluate the concentration and purity using absorbance readings at 260 and 280 nm. The concentration and purity ranged from 1.7 to 1.9 and 10 to 65 ng/µl, respectively. A Promega (USA) Quants TM Fluorometer is used for this test. RNA may be converted by CdnA from total RNA using a cDNA synthesis kit (ADD Bio/Korea).

Quantification Methods: Relative Strategies (The 2-ΔΔCt (Livak) Method)

The copy counts of the genes GADPH, FAS, and miRNA34a-5P were determined using the Ct values derived from the genomic DNA sample. Following that, these values were contrasted with the slopes and intercepts of the standard curves that had been previously created. Using the pertinent mathematical approaches, a detailed comparison of the formulae and ratios employed in the two quantification procedures (relative quantification) was conducted. For relative quantification, either 100% efficiency for all tests or the actual findings were used.

After the analysis of Ct values, for instance, several techniques might be used to compare the target gene's expression level in the test sample to that in the calibrator sample. The likelihood that both target and reference genes are amplified with comparable ~100% efficiency is measured using the most widely used and straightforward method. The target and reference genes should have the same amplification efficiencies, which is one of the presumptions that should be verified before using the Livak approach. The following formula was used to normalize transcript levels using GAPDH, miRNA34a-5P, and FAS data based on the delta-delta Ct technique.

$$\Delta Ct(test) = Ct\,(target, test) - Ct\,(ref, test)$$
$$\Delta Ct(control) = Ct\,(target, control) - Ct\,(ref, control)$$
$$\Delta\Delta Ct = \Delta Ct(test) - \Delta Ct(control)$$
$$2 - \Delta\Delta Ct = ratio\ of\ normalized\ expression$$

STATISTICAL ANALYSIS

Descriptive statistics showed a statistically significant correlation between the two groups' RT-qPCR amplification and data normalization for FAS, miRNA34a-5P, and the housekeeping gene (GAPDH) with a P-value less than 0.05. The findings of this research are shown as mean ± SD or percentages. A T-test was used to assess the mean differences between two groups for quantitative variables using SPSS version 27 (SPSS Inc., Chicago, IL, USA). If the P-value was less than 0.05 at a 95% CI, it was deemed statistically significant. GraphPad Prism version 9 (GraphPad Software, San Diego, CA, USA) was used to statistically analyze clinical biological indicators. The results were shown as mean ± standard deviation (SD) and $P \leq 0.05$. Examining the mean ± SD was made easier by the student's t-test. A p-value of less than 0.05 indicates statistical significance.

3. RESULTS AND DISCUSSION

Sample Clinical and Biochemical Features of Study Groups

These features studied the parameters tests of the control group (G1) and β-thalassemia major patients' group (G2), and they gave the values shown in Tables 1. β-TM refers to beta thalassemia major; FBS denotes fasting blood sugar; HbA1c indicates Glycated Hemoglobin; TC signifies total cholesterol; TG represents triglycerides; ALT stands for Alanine Transaminase; AST refers to Aspartate Aminotransferase; ALP denotes Alkaline Phosphatase; T. Protein indicates total protein; T. Bilirubin signifies Total Bilirubin; T4 represents thyroxine; TSH stands for Thyroid-Stimulating Hormone; HGB denotes Hemoglobin; and RBCs refer to Red Blood Cells. The data are shown as mean ± SD. A P-value of less than 0.05 is deemed significant, as determined by the T-test. Shah, F. et al. demonstrated that patients with β-TM had significantly higher ferritin levels than healthy controls in biomedical data. Serum ferritin is a surrogate for tissue iron status based on which organ damage due to iron overload is seen from repeated transfusion and low hepcidin. Iron homeostasis is impaired in thalassemia patients due to frequent blood transfusions as well as disturbances in the pathophysiology of iron metabolism characterized by low hepcidin and high endogenous skeletal muscle-derived erythroferrone (ERFE) resulting in increased iron absorption and mobilization [11]. Chang, Y.S. et al. demonstrated that patients with beta-thalassemia major (β-TM) have significantly higher FBS and HbA1c levels than healthy controls according to the available biomedical data. The excessive accumulation of ferritin leads to the oxidative stress, hepatic and cardiac fibrosis, and eventually the organ failure in the heart, liver, pancreas, and endocrine glands. Frequent blood transfusions cause iron overload, which is linked to altered fasting blood sugar. Excessive iron accumulation in the pancreas is also available, which may induce the death of β-cells, resulting in insulin resistance and diabetes [12]. Kumaravel et al. have demonstrated that biomedical data indicate higher triglyceride levels in patients with β-thalassemia major (β-TM) compared to healthy controls. Total cholesterol (TC) may decrease by increasing erythropoiesis, hemolysis, and transfusion of iron. TG impacts caused by chronic anemia, inflammation, iron overload, and liver injury Chronic hemolysis and oxidative stress disrupt lipid metabolism and increase triglycerides [13]. The authors showed that, according to biomedical data, patients with β-thalassemia major (β-TM) had higher ALT and AST levels relative to healthy controls. Chronic transfusion could lead to pancreatic injury and this could reduce lipase production and secretion. Elevated ALT may tell you that the liver is damaged due to iron deposition, hepatitis, drug, or blood transfusion. Conditions that can damage hepatocytes and consequently elevate AST levels include iron buildup, hemolysis, and viral diseases of the liver such as hepatitis. An AST/ALT ratio > 1 indicates liver fibrosis or cirrhosis and provides an additional perspective on liver health that differs from the AST/ALT ratio [14]. According to the authors For example, a biomedical data revealed higher ALP concentrations in β-TM than those in healthy controls. The liver iron increases with repeated blood transfusions and high levels of enzymes lead to poor liver damage. Iron overload causes hepatic and skeletal injury, resulting in the release of alkaline phosphatase (ALP) into the bloodstream. High ALP values in these people's bloods may signify osteocalcin and liver impairment [15]. A number of biomedical studies have indicated that albumin concentrations are decreased in beta-thalassemia major (β-TM) patients compared with healthy controls. Iron induced oxidative stress damages and dysfunctions all organ cells as shown by the Fenton reaction artesunate and iron reaction mechanism. Albumin levels might be diminished by splenomegaly but can be increased in iron overload and hepatitis infections. Poor prognosis due to severe illness and Hypoalbuminemia causing oedema and ascites. Increased globulin is associated with chronic inflammation, immunological activation, ineffective erythropoiesis-related liver injury, iron overload, and repeated transfusions [16]. The authors showed increased albumin/globulin ratio in patients with beta-thalassemia major (β-TM) compared with healthy controls, upon analysis of biomedical data. Increased albumin/globulin (A/G) ratio indicates chronic inflammation, immunological imbalance and hepatic dysfunction in

associations to high globulin and low albumin levels. Total protein reflects the amounts of both albumin and globulins and can be influenced by iron-related hepatopathy in beta-thalassemia. Niaz, A. et al. previously reported that biomedical data indicate lower total protein concentrations in β-thalassemia major (β-TM) patients compared to healthy controls [17]. Protein deficiency itself is often worsened by malabsorption and inadequate intake commonly observed in these patients due to associated gastrointestinal conditions (eg, lactose intolerance, vitamin and mineral deficiencies, and early satiety due to splenomegaly). The low total protein levels found in beta-thalassemia major are due to a combination of hepatic dysfunction, chronic inflammation, malabsorption, protein loss, and in some cases, endocrine disturbances [18]. Also, biomedical data showed higher total bilirubin levels in the subjects with beta-thalassemia major (β-TM) than healthy controls. Elevation in Bilirubin, Increased breakdown of erythrocytes, liver problems, e.g., iron overload disorder, hepatitis, or bile duct obstruction Gallstone is more prevalent with thalassemia because of the gallbladder bilirubin back-up. The excess hemolysis, insufficient erythropoiesis, and splenic egress of erythrocytes ultimately lead to high total bilirubin levels. Routine test of total bilirubin level ratio may be the signal of liver injury or biliary obstruction and predict disease severity [19]. Compared to healthy controls, demonstrated that bibliometric analysis of the studies confirmed that biomedicine showed that TSH was suppressed while T4 concentrations increased in beta-thalassemia major (β-TM) patients. Thyroid is an endocrine gland that absorbs excess iron creating free radicals and reactive oxygen species that lead to damage to thyroid cells and reduces the conversion of T4 to T3. The accumulation of iron in the pituitary gland could impair the production of TSH, leading to thyroid malfunction and other hormonal disturbances [20]. As demonstrated by the authors, the biomedical data evidenced lower concentration of creatinine and relatively higher blood urea level in β-thalassemia major (β-TM) patients as healthy controls. Superimposed on this is a resurgent iron overload caused by repeated blood transfusions, which can result in excess iron deposition in the kidneys and ultimately lead to renal impairment and reduced glomerular filtration and an increase in creatinine. Although chelation therapy can be nephrotoxic, causing an increase in serum creatinine, it controls iron overload. Iron overload from multiple blood transfusions can cause renal tissue damage and impaired waste filtration, likely leading to renal failure and increased blood urea levels [21]. Lal, A. et al. Furthermore, according to biomedical data, lower hemoglobin (HGB) levels in individuals with beta-thalassemia major (β-TM) relative to healthy controls. Regular transfusions are required in many patients with β-thalassemia major in order to keep Hb levels over 9.5 g/dL, with the transfusion frequency depending on the severity of the anemia and hemolysis. Expected hemo status requires regular transfusions, particularly in children. Decreased synthesis of beta-globin results in significant anemia, low hemoglobin concentrations (<7 g/dL), and manifestations including pallor, growth retardation, and skeletal deformities [22]. Sharif, Y. et al. reported that using biomedical data, they showed that individuals with β-thalassemia major (β-TM) have lower total red blood cell counts double than healthy controls. Imbalanced globin chain synthesis induces errant (or false) RBCs, hence ineffective erythropoiesis, hemolysis and low RBCs [23]

Association of Expression of FAS and miRNA34a-5P Genes as Compared with Housekeeping (GAPDH) Gene and Risk of β-TM Patients' Group (G2) Compared with Control Group (G1)

These features studied the gene expression of FAS and miRNA34a-5P of the control group (G1) and Patients with β-thalassemia major group (G2), and they gave the values shown in Table 2. Gene expression analysis of the two groups revealed a significant difference of FAS and miRNA34a-5P expression between G2 and G1 groups (P-value < 0.001). Beta thalassemia is classically thought of as a disorder primarily of ineffective erythropoiesis leading to anemia; here we identify FAS as playing a central role for the first time in the pathogenic mechanism of the disease. In beta-thalassemia, an excess of unpaired alpha globin chains results from a lack of functioning beta-globin chains. This leads to oxidative stress and harms the bone marrow's erythroid precursors. But this injury causes augmentation of FAS receptor in the erythroid cells. Fas-associated death domain (FADD) acts like a death receptor: It instigates a caspase-dependent apoptotic cascade upon interaction with FAS ligands (FasL), which is one of the causes of premature apoptosis of erythroblasts [24] FAS-provoked cell death also points to a reason for pathetic erythropoiesis and the lethal anemia of beta thalassemia patients. Thus, FAS expression on erythroid precursors is increased in the disease and contributes to the pathology [25]. The study investigates the role of miRNA-34a in beta-thalassemia along with an integrated signaling pathway coupling p53, miRNA-34a and SIRT1 in determining both erythroid cell death and globin gene expression. The latter involves p53 activation of miRNA-34a due to futile erythropoiesis and iron-mediated oxidative stress in beta-thalassemia leading to deacetylation and repression of SIRT1, and NAD^+-dependent deacetylase which under normal physiological conditions deacetylates and represses p53, followed by inhibition of miRNA-34a. Such inhibition initiates a positive feedback mechanism through elevated p53 activity, and facilitates erythroid progenitor cell apoptosis [26]. Furthermore, miRNA-34a reduces the expression of many apoptotic repressor genes including BCL2, resulting in excessive erythrocyte death. It also inhibits erythroid TFs such as KLF1 and NRF2 that indirectly modulate the transcription of γ-globin and β-globin genes. This ultimately leads to defective erythropoiesis, defective hemoglobin synthesis, and greater clinical severity. For this reason, miRNA-34a represents a downstream effector of cellular stress in beta-thalassemia and a candidate therapeutic target for the engineering of erythroid cell survival and globin expression [27]

Relative Quantification of FAS and miRNA 34a Genes as Compared with Housekeeping (GAPDH) Gene

Nucleic acid measurement is crucial for characterizing blood cell lines and documenting producer clones. Quantitative real-time RT-PCR is extensively used in many gene quantification methodologies, owing to its extensive dynamic range, good consistency, and high precision. While qPCR has significant promise for analytical applications, a comprehensive knowledge of its fundamental concepts is crucial. Relative calculations are made for real-time PCR findings [28]. Several methods for relative quantification of target gene transcripts versus an internal reference gene transcript were examined in this work, along with two sources of absolute quantification. The relative expression ratio was computed utilizing data analysis mathematical models that were based on the PCR efficiency and crossing point deviation of the examined transcripts. The transgene copy number was determined differently using several relative and absolute quantification techniques in this investigation [29], as shown in Table 3. The foundation of relative quantification is the comparison of a target gene's expression levels with those of a reference gene. The method, which compares a target gene's expression levels to those of a housekeeping gene (also known as a reference or control gene), is theoretically suitable for the majority of applications intended to investigate physiological variations in gene expression levels [30]. A range of real-time qPCR studies may be correlated with these relative results, which are represented by independent units. Obtaining the endogenous DNA sequence with known low copy counts per genome to act as the internal control is a crucial first step in real-time PCR analysis. To ensure detection sensitivity, the best choice for this estimate is a single-copy endogenous gene per genome. The single-copy internal control gene used for our experiment was GADPH [31]. Absolute quantification, on the other hand, determines the input template copy number using an internal or external calibration curve. Reference genes or internal controls are used in qPCR studies to normalize the data by taking into consideration differences in the quantity of cDNA utilized as templates. Excellent is a reference gene that consistently expresses itself in samples from different experimental circumstances or time periods. Six distinct relative quantification techniques were used to assess qPCR quantification techniques [32]. The presence of PCR inhibitors, temperature variations in plate-based equipment, and pipetting errors that lead to different amounts of master mix in reactions are some examples of variables that might cause variations in efficiency that are not related to calculation assumptions. The problem of variable input template amounts of the target gene is resolved by normalizing to a reference gene. Relative changes in gene expression may be computed using the 2-$\Delta\Delta Ct$ method [33]. The target and reference amplification efficiencies need to be nearly equal in order to verify the $\Delta\Delta Ct$. The utilization of data from the real-time PCR experiment to accomplish standardization is a noteworthy aspect of the 2-ΔΔCt technique. The two primary drawbacks of the 2-ΔΔCt approach are its reliance on the erroneous premise of 100% PCR efficiency and its indifference to background fluorescence, which results in inconsistent results. In a sample efficiency adjusted model, the expression of the target gene is normalized using the expression of a reference gene (REF), which is obtained from standard and often used reference genes. This strategy's primary flaw is that the drug being used alters the most often mentioned transcripts from so-called stably expressed housekeeping genes [34]. The average qPCR efficiency in LinRegPCR was determined using each of the distinct fluorescence curves for a single amplicon. The mathematical approach computes linear regression in the exponential part of the fluorescence curve using real-time PCR efficiency. The ratio between the reference and target genes, including the efficiency adjustment, was determined using a modified method based on previously published formulae. Without needing successive dilutions of the template, the approach may compute the Efficiency% result [35]. By not demanding that the PCR systems for the target and internal control genes have the same amplification efficiencies, the relative quantitation formula prevents potential bias from minute variations in amplification efficiency between the target and endogenous reference genes. The logarithm of the process's starting molecule input, the Ct value, and the threshold fluorescence are all linearly related. Therefore, any reaction components that become limited during the plateau period will not influence quantification, which will always take place during the exponential phase. The differences in efficiency generated by each method for each gene under investigation impacted the differences between the calculation methods [36]

Relative Quantification of Housekeeping (GAPDH) Gene Expression in the Study Groups (G1 and G2)

The amplification curve resulting from the qPCR reaction for the GAPDH gene in the control samples (blue) and the β-TM patients' samples (green) is shown in FIGURE 1a. The x-axis represents the number of cycles, while the y-axis represents the relative fluorescence units (RFU). The amplification curve shows the increase in fluorescence during the cycles of the qPCR reaction with the amplification of the target DNA, where the RFU increases with the increase in the amount of amplified DNA. Each curve represents an individual reaction, as the curves start at the baseline level and then enter the oscillatory amplification phase. The cycle threshold value (CT) represents the point where each curve intersects with the horizontal threshold line (green). The CT value is inversely proportional to the concentration of target DNA in the sample. Most of the curves showed clear exponential and plateau phases. Careful assembly of the curves indicates good production and a stable amount of primary template, which proves the accuracy and effectiveness of the amplification of the GAPDH gene in samples of the control (G1) and β-TM patients (G2). The appearance of the curve of one of the DNA samples in the β-TM patients was below the baseline, indicating that the CT value was high and that the concentration of target DNA was low. The amplification efficiency indicates the reliability of the gene expression results for the GAPDH gene in the samples of control (G1) and β-TM patients (G2), and thus it can be used as a reference gene for analyzing the normalization

data for other target genes in these samples. The melting curve resulting from the qPCR reaction of GAPDH in the control samples (blue) and the β-TM patients' samples (green) shows the presence of temperature on the x-axis, while the change in relative fluorescence units with temperature ((-d (RFU)/dT)) is on the y-axis, as in FIGURE 1b. The blue lines indicate the melting curves of GAPDH in the control group samples (G1), while the green lines indicate the melting curves of GAPDH in the β-TM patients' samples (G2). The precise clustering of the peaks indicates the specificity of the qPCR amplification products, as the peaks appear as a single peak in most samples, indicating the homogeneity of the peak shape and location, and thus indicating high specificity for analyzing normalization data and a minimum level of contamination, as most samples showed clear and specific amplification. Each curve represents the PCR reaction of a single sample, and the peak indicates the temperature at which the double-stranded DNA melts or decomposes into single strands. The absence of double-shouldered bands indicates the absence of non-specific products or formation of primer dimers, which indicates the quality and specificity of the amplification in the qPCR reaction and indicates the accuracy of the gene expression results for the GAPDH gene in samples of control (G1) and β-TM patients (G2). Therefore, the GAPDH gene is considered the reference and internal control gene in PCR reactions to normalize the data of other target genes in samples of control (G1) and β-TM patients (G2).

Relative Quantification of FAS Gene Expression as Compared with Housekeeping (GAPDH) Gene in the Study Groups (G1 and G2)

As provided in FIGURE 2a, the expression of the FAS gene in β-TM patients is significantly lower than that in the control group. The mean ± SD values were 0.8029± 0.04881 for patients with β-TM (G2) and 1.729± 0.1594 for controls (G1). Statistical analysis performed in GraphPad Prism using a t-test (T-test = 5.556) shows the difference is highly significant ($P < 0.001$) are shown in TABLE 2 and FIGURE 2a. The amplification curves resulting from the qPCR reaction used to measure gene expression of the FAS gene using the GAPDH gene as a reference gene in DNA samples from the control group (G1), whose amplification curves appear in yellow, and the β-TM patients' group (G2), whose amplification curves appear in red, are shown in FIGURE 2b. The x-axis represents the number of cycles in the qPCR reaction, while the y-axis represents the relative fluorescence units (RFU), which indicate the amount of amplified DNA. The horizontal green line represents the detection threshold, which characterizes the clarity and significance of the signal and from which the CT value can be calculated. The gene expression of FAS gene was low in β-TM patient samples, indicating a disorder in the mechanisms of programmed death. The amplification curves of β-TM patient samples (red) showed intersections with the threshold line in early cycles (25 to 40 cycles), indicating a high CT value. This confirms the presence of a decrease in the initial concentrations of DNA samples, indicating a low level of gene expression of the FAS gene in β-TM patient samples (G2). On the contrary, the amplification curves of the control samples (yellow) intersected with the threshold line in late cycles (22 to 38 cycles), indicating a decrease in CT values. This confirms the presence of an increase in the initial concentrations of DNA samples, indicating a high level of gene expression of the FAS gene in the control samples (G1). These results confirm that the mRNA transcripts of the FAS gene in the β-TM group (G2) were relatively lower after normalization than in the control group (G1), indicating a significant decrease in the gene expression of the FAS gene in cells of β-TM patients. This indicates that the effect of β-TM disease causes a disruption in the regulation of the apoptosis pathway and programmed cell death mechanisms, which contributes to the accumulation of abnormal and damaged RBCs inside the bone marrow and leads to bone marrow enlargement. Consequently, the spleen works to filter and dispose of them, leading to splenomegaly. Therefore, decreased expression of the FAS gene in β-TM patients can be considered a biomarker for detecting the β-TM disease and determining the response to treatment in patients. The melting curve represents the result of the qPCR reaction analysis of the expression of the FAS gene using the GAPDH gene, an internal reference gene, in DNA samples from the control group (G1), whose curves appear in yellow, and the β-TM patients' group (G2), whose curves appear in red, as shown in FIGURE 2c. The melting curve shows the specificity of the amplification of the FAS gene by studying the melting points of the products located on the x-axis (temperatures) and the change in relative fluorescence units with temperature change (−d(RFU)/dT), which are located on the y-axis. The melting curve shows the presence of a single sharp and symmetrical peak at a temperature ranging from 78 to 82°C for both the control (yellow curves) and the β-TM patients (red curves), indicating that the resulting amplification is highly specific and specific to a single product, without the presence of non-specific products or the formation of primer-dimers, which indicates the success and efficiency of the reaction. The similarity in the melting temperatures (TM) between groups (G1 and G2) indicates the quality of the amplified DNA structure. The reliability of the FAS gene expression results calculated from the amplification curves confirms the specificity of FAS gene amplification in both groups. The appearance of a single, clear, and identical peak in both control samples and β-TM patients indicates the absence of unwanted by-products, which confirms the accuracy of the reaction and the absence of interferences and impurities. This confirms that the differences in FAS gene expression between the two groups are due to real differences in the amount of mRNA and not to technical errors, which enhances the accuracy of FAS gene amplification and confirms the reliability of the quantitative gene expression results in Table 3.37, which found that the efficiency of FAS gene amplification in control samples and β-TM patients ranged between 90 and 96%. This confirms the importance of using FAS gene expression results in the accurate diagnosis of β-TM disease.

Relative Quantification of miRNA34a-5P Gene Expression as Compared with Housekeeping (GAPDH) Gene in the Study Groups (G1 and G2)

As shown in FIGURE 3a, the miRNA34a-5P miRNA gene expression level in the β-TM patients is much lower than that in the control group. Patients with β-TM (G2) showed a reduced average ± SD level of expression of 0.7185± 0.08199 while controls (G1) showed an average of 1.386± 0.1072. Statistical analysis performed in GraphPad Prism with a T-test (T-test = 4.945) shows the difference is highly significant ($P < 0.001$). This chart shows the gene expression for miRNA34a-5P (FIGURE 3a). The amplification curves resulting from the qPCR reaction to study the expression of the miRNA34a-5P gene in samples taken from two study groups: the control group (G1), represented by the green curves, and the β-TM patients' group (G2), represented by the red curves, using the reference gene GAPDH to control gene expression, as shown in FIGURE 3b. The x-axis represents the number of cycles required for amplification, while the y-axis represents the relative fluorescence units (RFU), which reflect the amount of product multiplied during the reaction. The horizontal green line represents the threshold line separating the sample amplification curves from the background noise; at it, the CT value is calculated, and the fluorescence signal begins to exceed the background. The control group samples (G1) begin to amplify at a higher number of cycles, indicating a decrease in miRNA34a-5P gene expression in this group. The green amplification curves for the control samples begin to rise, exceeding the threshold at (22 and 32 cycles), indicating the beginning of amplification of the control samples late, indicating that the gene expression of the miRNA34a-5P gene is relatively low because the number of amplification cycles required to detect it is high (the CT value is higher). While the samples of the β-TM group (G2) start to amplify at a lower number of cycles, which shows an increase in the expression of the miRNA34a-5P gene in this group, as the red amplification curves of the β-TM samples are early to rise and exceed the threshold, and they start at (20 and 36 cycles), which indicates that the amplification of the β-TM samples starts at an early time and indicates that the gene expression of the miRNA34a-5P gene is relatively high in these samples because the number of amplification cycles required to detect it is lower (the CT value is lower). The green curves, which represent the amplification of the control samples (G1), exceed the threshold line at a higher number of cycles (the CT value is high), indicating that the expression of the miRNA34a-5P gene in the control group (G1) was lower than its expression in the β-TM patients group (G2), because the red curves, which represent the amplification of the β-TM patients' samples (G2), were early in exceeding the threshold line at lower cycles (the CT value is low), which indicates an increase in the gene expression of the miRNA34a-5P gene. In the β-TM patient group (G2), the increased gene expression of the miRNA34a gene in β-TM patients indicates its regulatory role in the biological processes related to β-TM disease, because the miRNA34a-5P gene is a regulatory part of the microRNA genes, which participates in regulating gene expression for the process of programmed cell death or the response to oxidant stress. Therefore, its increased expression in β-TM patients occurs due to an increase in the regulation of the miRNA34a-5P gene, the response of the disease to the state of chronic anemia, and an imbalance in the regulatory gene pathway specific to microRNAs in β-TM disease. The use of GAPDH as an internal control gene to adjust for differences in total RNA amounts, reverse transcription efficiency, and ΔCT determination between miRNA34a-5P and GAPDH, which are used in the ΔΔCT method to calculate relative expression, indicates the specificity and limitation of amplification products and indicates the quality and accuracy of the reaction. The higher level of gene expression of the miRNA34a-5P gene in β-TM patient samples (G2) compared to the control (G1) makes miRNA34a a biomarker that can be used for early detection of β-TM disease and to evaluate disease progression or treatment effectiveness in β-TM patients. Melting curve analysis resulting from the qPCR reaction for the study of miRNA34a-5P gene expression using the GAPDH gene, an internal reference gene, in samples from the control group (G1), whose curves are shown in green, and the β-TM patient group (G2), whose curves are shown in red, is shown in FIGURE 3c. The x-axis represents the temperature in Celsius that is gradually raised to conduct the melting curve analysis, while the y-axis represents the change in relative fluorescence units with changing temperature (−d(RFU)/dT), which represents the rise or fall of the fluorescence signal as a result of the separation of the two DNA strands (the melting of the product), while the green horizontal line represents the melting threshold by which the melting peaks are identified and the concentrations of the product formed are known by determining the melting temperature (Tm) value. The appearance of a single sharp and defined melting peak located in a temperature range at (83 and 88 °C) in the study groups (G1 and G2) indicates the specificity of the reaction results in a single, specific product due to the absence of non-specific products or the formation of dimer-primers, which appear as additional peaks at low temperatures (low Tm). The melting curves of the control group (G1), which appear in green, showed higher, more regular, and more homogeneous peaks in terms of intensity and location, indicating a higher level of miRNA34a-5P expression, its stability, and the presence of a balance in the expression of the gene across the control group samples (G1); this is evidenced by the regularity of the peaks. The melting curves of the β-TM patient group (G2) appear in red, with lower, more distinct, and lower peaks in intensity, indicating a lower level of miRNA34a-5P expression and variation in its expression between the β-TM patient samples, indicating molecular changes in the response or genetic regulatory mechanisms due to the presence of disturbances in the gene expression of regulatory miRNAs molecules in β-TM patients. The differences in gene expression of the miRNA34a-5P gene between the study groups (G1 and G2) are due to real differences in the amount of mRNA because the use of GAPDH as an internal control reduces technical errors in the qPCR reaction, which enhances the accuracy of the amplification of the miRNA34a-5P gene and confirms the reliability of the quantitative gene expression results shown in Table 3.37, which found that the amplification efficiency of the miRNA34a-5P gene in samples from the control (G1) and β-TM patients groups (G2) ranged from 95 to 98%. This confirms the importance of using the gene expression results of the miRNA34a-5P gene in the accurate diagnosis

of β-TM disease, because the low gene expression of the miRNA34a-5P gene, which is considered a regulatory gene associated with the pathways of cellular stress and apoptosis, which makes the miRNA34a-5P gene a biomarker for the detection of β-TM disease and the therapeutic response in β-TM patients.

4. Tables and Figures

A. Tables

Table 1. The Clinical and Biochemical features of (G1 and G2) groups in this study

Parameters	*Groups*		*T-test*	*P-value*
	G1-control Mean ± SD	G2-, β-TM Mean ± SD		
Ferritin (ng/ml)	139.6± 33.29	4921± 1072	42.28	0.0001*
FBS (mg/dl)	94.21± 10.62	166.8± 18.33	32.49	0.006*
HbA1c (mmole/mol)	5.43± 0.32	7.60± 0.55	32.50	0.015*
TC (mg/dl)	142.27± 34.04	105.67± 26.9	8.006	0.03*
TG (mg/dl)	92.17± 24.31	200.13± 22.35	31.018	0.018*
Lipase	39.20± 0.9172	43.18± 0.3468	4.057	0.042*
ALT (IU/L)	20.66± 7.491	54.57± 9.946	25.837	0.005*
AST (IU/L)	25.56± 8.21	78.29± 17.64	25.719	0.007*
ALP (IU/L)	100.49± 28.36	197.49± 32.46	21.350	0.003*
AST/ALT ratio	0.962± 0.038	0.915± 0.028	0.992	0.323
Albumin (g/dl)	4.390± 0.573	4.142± 0.437	3.795	0.3725
Globulin (g/dl)	2.990± 0.776	0.638± 0.409	23.78	0.037*
Albumin/Globulin ratio	1.490± 0.063	9.291± 1.254	6.286	0.021*
T. Protein (g/dl)	7.38± 0.605	4.78± 0.503	29.84	0.035*
T. Bilirubin (mg/dl)	0.779± 0.143	6.422± 1.78	19.26	0.018*
T4 (µg/dl)	7.97± 1.95	2.77± 1.005	22.467	0.01*
TSH (µIU/mL)	2.95± 1.17	7.75± 1.52	23.740	0.01*
Creatinine (mg/dl)	0.813± 0.154	0.453± 0.074	20.006	0.021*
B. Urea (mg/dl)	14.19± 3.75	42.21± 8.57	28.417	0.013*
HGB (g/dl)	14.61± 1.56	6.00± 0.935	24.98	0.015*
RBCs (µLx 10^6)	5.21 x 10^6± 0.614	2.47 x 10^6± 0.823	25.312	0.009*

The mean ± SD of β-TM (G2), and control (G1) are compared in Table 1. The P value indicates that rate of measurements calculated for β-TM (G2), the Healthy (G1) expressed the mean ± SD values. As seen (Table 1), when the two selected groups were different concerning the mean values of each of the parameters (FBS, HbA1c, ferritin, TC, TG, lipase, ALT, ALP, AST, total bilirubin, globulin, albumin/globulin ratio, total protein, T4, TSH, creatinine, blood urea, HGB, and RBCs), the differences were significant as their P-values were influential [P-value <0.05] in the β-TM patients' group (G2) when compared with the control group (G1).

TABLE 2. The comparison between the gene expression of FAS, miRNA34a-5P genes as compared with housekeeping (GAPDH) gene of the (G1 and G2) groups in this study

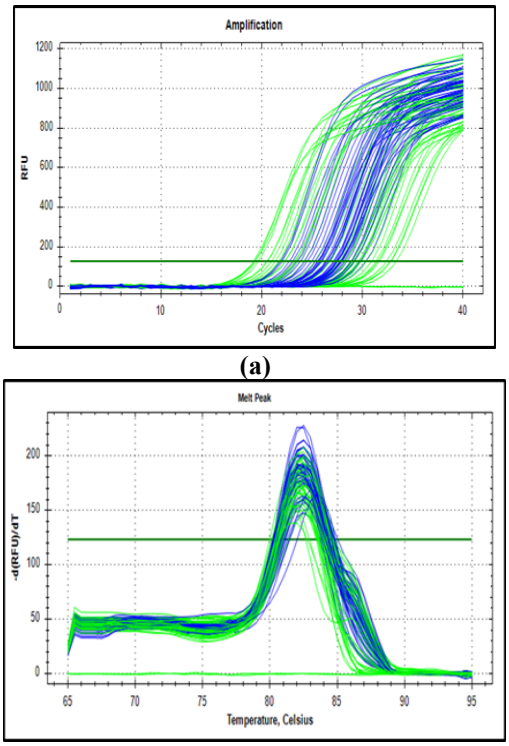
Genes	**Groups**		**T-test**	**P-value**
	G1-control Mean ± SD	G2-, β-TM Mean ± SD		
FAS	1.729± 0.312	0.8029± 0.1631	5.556	0.001*
miRNA34a-5P	1.386± 0.1017	0.7185± 0.1778	4.945	0.001*

The expression of genes FAS and miRNA34a-5P is presented on Table 2. This total gene expression of FAS and miRNA34a-5P for the patient beta-thalassemia group G2 and compared to control group G1. Group comparison of βTM patients (G2) and control group (G1) with P-value < 0.05, values show mean ± SD is depicted in Table 2.

TABLE 3. Relative Quantification of FAS and miRNA34a-5P Genes as Compared with Housekeeping (GAPDH) Gene of the (G1 and G2) groups in this study**.**

Gene (GADPH, ref)	Avg CT-G1	Avg CT-G2	ΔCT-G1	ΔCT-G2	ΔΔCT	$2^{-\Delta\Delta CT}$	Efficiency%
FAS	26.0	32.7	2.0	7.7	5.7	0.017	90–96%
miRNA34a-5P	26.1	26.7	2.1	1.7	0.4	1.32	95-98%

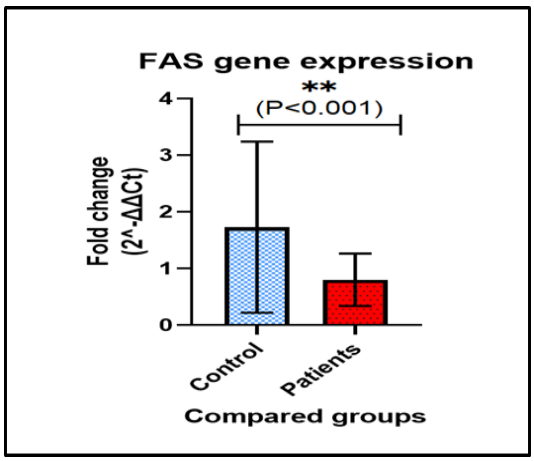
B. Figures



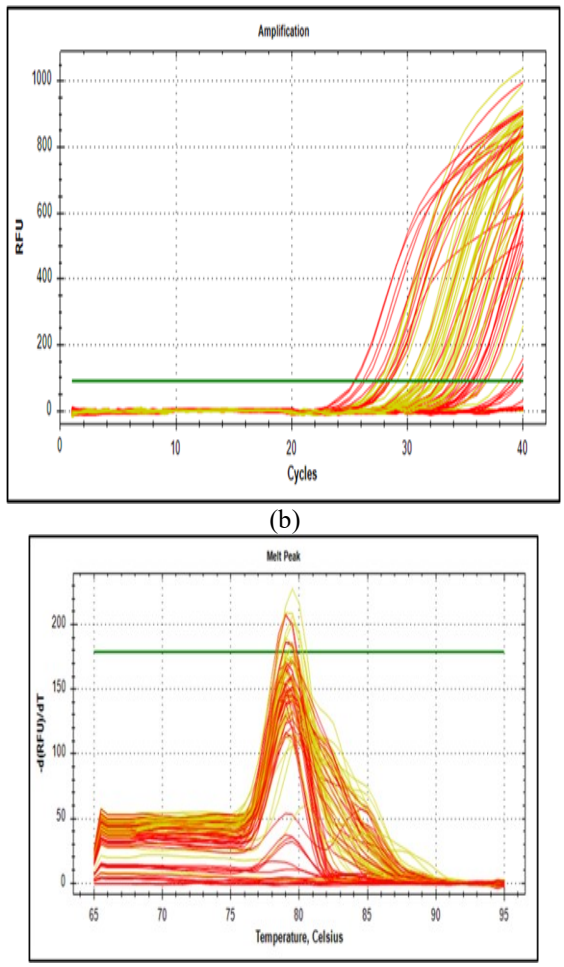
(a)

(b)

Figure 1. (a): Amplification curve of housekeeping (GAPDH) gene expression in β-TM patients (G2, green line) and control individuals (G1, blue line). The crossing threshold (CT) obtained in cycles and relative fluorescence units (RFU) was found to be associated with the presence of successful amplification curves. (b): Disassociation melting curve analysis of housekeeping (GAPDH) gene expression in β-TM patients (G2) (Green) and control (G1) (Blue). Successfully dissociation curves with unique peak.



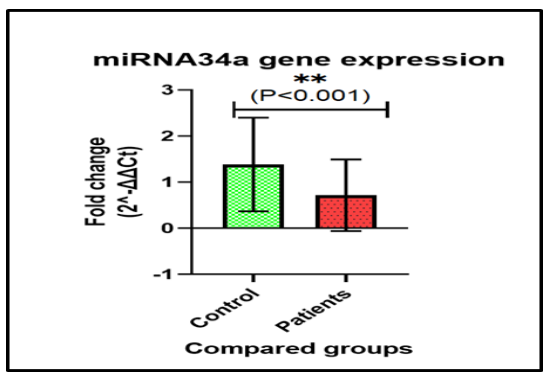
(a)



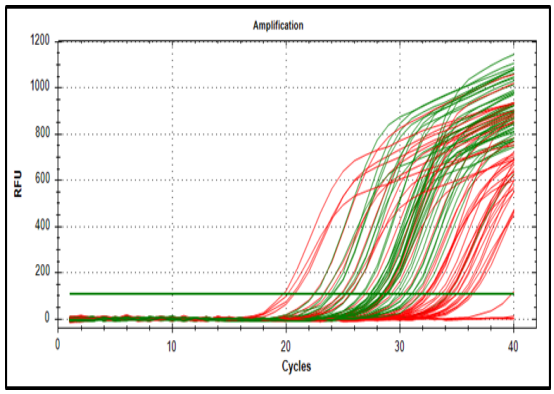
(b)

(c)

Figure 2. (a): 2-ΔΔCt comparison of FAS Gene Expression (G2 for β-TM patients, G1 for Control). (b): Amplification curve of the expression of the gene of interest (FAS) in β-TM patients (G2) depicted in red. Yellow indicates the comparison group (G1). By plotting the RFU over the number of cycles, the successful amplification curves show directly how the relevant crossing threshold (CT) varies with cycles. (c): Disassociation melting curve analysis with the samples tested for the gene of interest (FAS) in β-TM cases (G2) (Red) and controls (G1) (Yellow). For example, the plots of dissociation with a single peak being successfully fitted.



(a)



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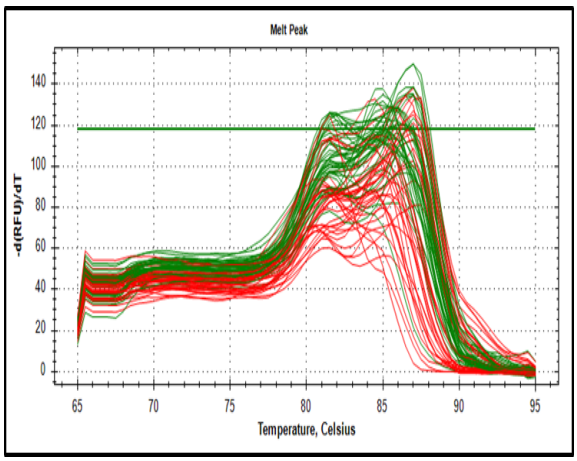

(c)

FIGURE 3. (a): 2-ΔΔCt comparison of Gene expression of miRNA34a-5P in β-TM patients (G2) and control (G1). (b): β-TM patients (G2) expression of miRNA34a-5P amplification curve (red) and control (G1) (green). Successful amplification curves are illustrated with the corresponding crossing threshold (CT) curve in relation to cycle numbers with round forming units (RFU) on the Y-axis. (c): Dissociation melting curve analysis of samples that were tested for expression of miRNA34a-5P gene inβ-TM patients (G2) (Red) and control (G1) (Green) With the single peak successful disassociation curves as shown.

5. CONCLUSIONS

According to the findings of this investigation, we conclude that the parameters (FBS, HbA1c, ferritin, TC, TG, lipase, ALT, ALP, AST, total bilirubin, globulin, albumin/globulin ratio, total protein, T4, TSH, creatinine, blood urea, HGB, and RBCs), along with the gene expression of FAS and miRNA34a-5P, serve as excellent diagnostic markers for patients with β-Thalassemia Major, given their significant P-value ($P < 0.05$). The amplification was excellent when looking at the gene expression of the Fas and microRNA genes, with amplification efficiencies of $90 - 110\%$ for the FAS gene and 95–98% for the miRNA34a-5P gene; this indicates that we can accurately diagnose the gene expression of these genes and their impact on beta thalassemia patients. Real-time reverse transcriptase PCR is an excellent method for studying gene expression.

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AUTHORS' DECLARATION:

We confirm that all the Figures and Tables in the manuscript belong to the current study

CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest regarding the publication of this study.

REFERENCES

[1] D. C. Baird, S. H. Batten, and S. K. Sparks, "Alpha- and beta-thalassemia: rapid evidence reviews," *Am. Fam. Physician*, vol. 105, no. 3, pp. 272–280. 2022.

[2] M. M. Salih, A. G. Al-Ziaydi, and A. H. H. Alzamili, "The Effect of Ferritin Level and Gene Expression of β-globin Promoter with β-thalassemia Patients in Al-Qadisiyah Governorate, Iraq," *J. Appl. Hematol*, vol. 14, no. 4, pp. 257–262. 2023.

[3] S. Daar *et al.*, "Mortality and complications in Omani patients with beta-thalassemia major: a long-term follow-up study," *Acta Bio Medica Atenei Parm*, vol. 94, no. 4. 2023.

[4] S. M. Alharbi, S. S. Alshammari, H. A. Alsiaf, and F. S. Alkhowaiter, "Systematic review of metabolic and endocrinological complications in beta-thalassemia major: insights from 2021 to 2023," *Int. J. Med. Dev. Ctries*, vol. 8, no. 1, p. 288.

[5] O. Raducka-Jaszul, D. M. Bogusławska, N. Jędruchniewicz, and A. F. Sikorski, "Role of extrinsic apoptotic signaling pathway during definitive erythropoiesis in normal patients and in patients with β-thalassemia," *Int. J. Mol. Sci*, vol. 21, no. 9, p. 3325. 2020.

[6] E. Noulsri and S. Lerdwana, "Reducing erythroblast apoptosis in β-thalassemia via unfolded protein response (UPR) signaling," *Med. Hypotheses*, vol. 177, p. 111117. 2022.

[7] K. Settakorn *et al.*, "Effects of green tea extract treatment on erythropoiesis and iron parameters in iron-overloaded β-thalassemic mice," *Front. Physiol*, vol. 13, p. 1053060. 2022.

[8] F. Wang, L. Ling, and D. Yu, "MicroRNAs in β-thalassemia," *Am. J. Med. Sci*, vol. 362, no. 1, pp. 5–12. 2022.

[9] S. S. Das *et al.*, "MicroRNA expression patterns in HbE/β-thalassemia patients: The passwords to unlock fetal hemoglobin expression in β-

hemoglobinopathies," *Blood Cells, Mol. Dis*, vol. 87, p. 102523. 2021.

[10] N. El-Khazragy *et al.*, "Circulating miRNAs and tissue iron overload in transfusion-dependent β-thalassemia major: novel predictors and follow-up guide," *Ann. Hematol*, vol. 100, no. 12, pp. 2909–2917. 2021.

[11] F. Shah *et al.*, "Relationship between Serum Ferritin and Outcomes in β-Thalassemia: A Systematic Literature Review," *J. Clin. Med*, vol. 11, no. 15, p. 4448. 2022.

[12] Y. S. Chang, L. Y. Hsiao, C. Y. Lin, M. C. Shih, M. C. Hsieh, and J. G. Chang, "Fasting glucose-to-HbA1c ratio is a good indicator of G6PD deficiency, but not thalassemia, in patients with type 2 diabetes mellitus," *Clin. Chim. Acta*, vol. 506, pp. 9–15. 2023.

[13] K. S. Kumaravel, D. Sampathkumar, P. Punitha, V. Anurekha, M. Hema, and B. Rameshbabu, "Premature atherosclerosis in children with transfusion-dependent thalassemia: a twin-center cross-sectional study," *Indian Pediatr*, vol. 59, no. 12, pp. 925–928. 2022.

[14] P. Ricchi *et al.*, "Liver steatosis in patients with transfusion-dependent thalassaemia," *Br. J. Haematol*, vol. 204, no. 6, pp. 2458–2467. 2023.

[15] P. Mahakkanukrauh, A. Sinthubua, and S. Das, "Thalassemia Major and Bone Loss: Things to Ponder," *Int. Med. J*, vol. 27, no. 2, pp. 185–187. 2022.

[16] G. S. Abdelmotaleb, O. G. Behairy, S. Hassan, and R. A. Khashaba, "Serum Levels of Ischemia Modified Albumin in Children with B-Thalassemia Major," *Benha Med. J*. 2023.

[17] A. Niaz, K. Tauseef, M. Zahid, H. Zafar, A. Nafisa, and N. Ikram, "Post Splenectomy Protein C Levels in Beta Thalassaemia Major Patients," *J. Haematol. Stem Cell Res*, vol. 2, no. 2, pp. 85–89. 2021.

[18] A. Ahmadi *et al.*, "Natural Anticoagulant Protein Levels in Patients With Beta-Thalassemia Major: A Case-Control Study," *J. Hematol*, vol. 13, no. 1–2, p. 23. 2022.

[19] K. S. A. A. Neaimy, M. M. K. Alkhyatt, and I. A. Jarjess, "New Insights of Oxidative Stress and Thalassemia May Lead to Antioxidant Therapy," *Pharmacogn. J*, vol. 16, no. 1. 2024.

[20] B. Akıncı, F. D. Yenigurbuz, A. Üstyol, and D. Ökdemir, "Thyroid Dysfunction in Beta-Thalassemia Major: Is It Related to Autoimmunity or Iron Overload?" *Acıbadem Üniversitesi Sağlık Bilim. Derg*, vol. 14, no. 3, pp. 377–382. 2023.

[21] A. Lal *et al.*, "The transfusion management of beta thalassemia in the United States," *Transfusion (Paris).*, vol. 61, no. 10, p. 3027. 2021.

[22] P. Z. Romadhon *et al.*, "Markers of Renal Complications in Beta Thalassemia Patients with Iron Overload Receiving Chelation Agent Therapy: A Systematic Review," *J. Blood Med*, pp. 725–738. 2023.

[23] Y. Sharif *et al.*, "Assessment of patients with β-thalassemia major, undergoing tertiary care at a regional thalassemia center in Pakistan," *Pak. J. Zool*, vol. 53, no. 1, p. 245. 2022.

[24] K. Trakarnsanga, N. Thongsin, C. Metheetrairut, C. Tipgomut, S. Poldee, and M. Wattanapanitch, "Genetic correction of haemoglobin E in an immortalised haemoglobin E/beta-thalassaemia cell line using the CRISPR/Cas9 system," *Sci. Rep*, vol. 12, no. 1, p. 15551. 2022.

[25] M. Sanchez-Villalobos, M. Blanquer, J. M. Moraleda, E. J. Salido, and A. B. Perez-Oliva, "New insights into pathophysiology of β-thalassemia," *Front. Med*, vol. 9, p. 880752. 2022.

[26] H. M. Sharshera, S. M. E. Maghraby, S. A. Aglan, I. L. Mikhael, and O. A. Balbaa, "Estimated Level of Plasma Micro RNA-210 in Beta Thalassemia Patients and Its Relation to Hemoglobin F Level and Disease Severity," *J. Appl. Hematol*, vol. 14, no. 3, pp. 208–213. 2022.

[27] M. Rahaman *et al.*, "Exploring the crosstalk between long non-coding RNAs and microRNAs to unravel potential prognostic and therapeutic biomarkers in β-thalassemia," *Mol. Biol. Rep*, vol. 49, no. 7, pp. 7057–7068. 2022

[28] L. Conceição Braga, B. Ô. P. Gonçalves, P. L. Coelho, A. L. Silva Filho, and L. M. Silva, "Identification of best housekeeping genes for the normalization of RT-qPCR in human cell lines," *Acta Histochem*, vol. 124, no. 1, p. 151821. 2022.

[29] Y. Li, Q. Ma, X. Shi, W. Yuan, G. Liu, and C. Wang, "Comparative transcriptome analysis of slow-twitch and fast-twitch muscles in dezhou donkeys," *Genes (Basel*, vol. 13, no. 9. 2022.

[30] L. Wang, Y. Xie, W. Chen, Y. Zhang, and Y. Zeng, "miR-34a regulates lipid droplet deposition in 3T3-L1 and C2C12 cells by targeting LEF1," *Cells*, vol. 12, no. 1, p. 167. 2023.

[31] Y. P. Chen, C. C. Hu, S. Tsai, Z. H. Wen, and C. Lin, "Identification of housekeeping gene for future studies exploring effects of cryopreservation on gene expression in shrimp," *Sci. Rep*, vol. 15, no. 1, p. 11046. 2023.

[32] A. Formosa *et al.*, "Validation of reference gene stability for miRNA quantification by reverse transcription quantitative PCR in the peripheral blood of patients with COVID-19 critical illness," *PLoS One*, vol. 18, no. 8, p. 286871. 2023.

[33] Y. Wang, F. Zhou, M. Li, Y. Zhang, N. Li, and L. Shao, "MiR-34a-5p promotes hepatic gluconeogenesis by suppressing SIRT1 expression," *Exp. Cell Res*, vol. 420, no. 1, p. 113336. 2022.

[34] H. T. Ong, C. M. Prêle, and R. J. Dilley, "Using RNA-seq to identify suitable housekeeping genes for hypoxia studies in human adipose-derived stem cells," *BMC Mol. cell Biol*, vol. 24, no. 1, p. 16. 2023.

[35] A. D. Singh *et al.*, "Identifying stable reference genes in polyethene glycol precipitated urinary extracellular vesicles for RT-qPCR-based gene expression studies in renal graft dysfunction patients," *Transpl. Immunol*, vol. 75, p. 101715. 2023.

[36] R. R. Su, Z. Y. Huang, C. W. Qin, X. L. Zheng, W. Lu, and X. Y. Wang, "Evaluation of reference genes in Glenea cantor (Fabricius) by using qRT-PCR," *Genes (Basel*, vol. 12, no. 12. 2021.