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# Expression of the FAM132B Gene in Iranian Patients with Beta-Thalassemia

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## ABSTRACT

**Background:** Iron homeostasis is a complex process involving multiple factors. Erythroblasts secrete erythroferrone (ERFE), which affects hepcidin production, thereby enhancing iron uptake. This study aimed to investigate the expression of the ERFE-encoding FAM132B gene in Iranian patients with beta-thalassemia major (BTM) and beta-thalassemia intermedia (BTI).

**Materials and Methods:** A total of 40 BTM and BTI patients and 20 healthy blood donors as a control group were recruited. Total RNA was extracted from whole blood samples, and cDNA was synthesized. Gene expression was quantified using a SYBR Green-based real-time PCR (RT-PCR) assay. Data analysis was performed by the GraphPad Prism program using the one-way ANOVA test.

**Results:** The expression of the FAM132B gene was upregulated in BTI patients compared to BTM patients ($p$ = 0.0151). Despite the higher mRNA fold change in BTI patients, no significant difference was observed in the FAM132B gene expression between beta-thalassemia patients (major and intermedia) and the control group.

**Conclusion:** The expression of the FAM132B gene was different between beta-thalassemia major and intermedia patients. Further studies should be conducted to better elucidate the role of erythroferrone as a potential therapeutic target in patients with beta-thalassemia.

**Keywords:** Beta-thalassemia; Gene expression; Erythroferrone (ERFE); FAM132B; Iron overload

## INTRODUCTION

Beta (β)-thalassemia is considered one of the most common genetic disorders worldwide[1,2], particularly in the countries of the Mediterranean basin[3]. In Iran, there are over 17,000 individuals diagnosed with beta-thalassemia[4], and the prevalence rate of β-thalassemia carriers is 4%, ranging from less than 2.0% to more than 8.0%[5]. Anemia is the most prominent feature of the disease, resulting from ineffective erythropoiesis in the bone marrow[2]. According to the severity of anemia, beta-thalassemia is categorized into three types: major, intermedia, and minor[6]. Blood transfusion gives thalassemia patients the chance of living longer, but this primary therapeutic regimen also has harmful consequences[7].

Although iron plays a crucial role in biological processes, its accumulation can be toxic. Therefore, iron overload is one of the primary complications of β-thalassemia, resulting from regular blood transfusions and increased intestinal iron absorption. The latter is a result of anemia[8]. Although iron-chelating therapy has improved the quality of life, iron overload remains the leading cause of mortality in thalassemia patients[9].

In humans, there is no mechanism for excreting excess iron, and iron homeostasis is regulated by absorption through several factors[10]. Erythroferrone (ERFE), encoded by the FAM132B gene[11] located on chromosome 2q37.3[12], has been described as a hepcidin repressor due to erythropoietin (EPO) stimulation[13]. This hormone is produced in the bone

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marrow by erythroblasts and increases the amount of iron available for hemoglobin synthesis by suppressing hepcidin production[11,13]. Therefore, erythroferrone plays a crucial role in iron metabolism and the treatment of various iron-related diseases[11,13,14].

Positive correlations between serum ERFE and erythropoietin have been reported in patients with chronic kidney disease[15]. It was shown that patients with iron deficiency anemia had higher serum erythroferrone levels compared to the healthy control group. Furthermore, in a study in Egypt, Saad et al. demonstrated that the serum erythroferrone level was significantly higher in beta-thalassemia patients (major and intermedia) compared to healthy individuals[16]. There was also a negative association between serum erythroferrone concentrations and hemoglobin levels, serum iron, transferrin saturation (TS%), and serum ferritin in patients with iron deficiency anemia[17]. Investigations in the mouse model showed that erythroferrone contributes to hepcidin suppression during malarial anemia[18]. Kautz et al. found that the severity of anemia of inflammatory diseases was higher in mice lacking ERFE[11]. In the mouse model of thalassemia, ablation of ERFE reduced iron overload by decreasing hepcidin to a normal level[19].

In addition to erythroferrone, hepcidin, as the most well-known regulator of iron metabolism, is predominantly expressed in hepatic tissue and encoded by the HAMP (hepcidin antimicrobial peptide) gene[20]. It acts by internalizing and degrading ferroportin (FPN), the only recognized exporter of cellular iron in vertebrates, leading to the retention of iron in macrophages and reduction of plasma iron concentration[21]. The deregulation of hepcidin can lead to various iron-related disorders, such as anemias or iron overload diseases[22].

Except for El-Gamal et al.'s study[23], the FAM132B gene expression has been analyzed in only a few studies, all of which have been conducted in beta-thalassemia intermedia mice[13,19]. To date, no investigation has been conducted into the expression of the ERFE-encoding FAM132B gene in Iranian beta-thalassemia patients; the present study is the first to do so. The purpose of this study was to investigate the expression of the FAM132B gene in patients suffering from beta-thalassemia major (BTM) and beta-thalassemia intermedia (BTI) in Iran.

## MATERIALS AND METHODS

### Patients and data collection

In this cross-sectional study, a total of 40 β-thalassemia patients were recruited from the Zafar Adult Thalassemia Clinic in Tehran between June 2018 and September 2018, comprising 20 patients with β-thalassemia major and 20 with β-thalassemia intermedia. All beta-thalassemia major patients were transfusion-dependent (1-2 per month). The following demographic and laboratory data were extracted from patients' histories using a prepared checklist: sex, age, hemoglobin value (g/dl), and serum ferritin level (ng/ml). Iron overload status was characterized by a ferritin level exceeding 1000 µg/ml, as detailed by the Thalassaemia International Federation (TIF)[24]. Twenty healthy blood donors were included as a control group. These donors had referred to the General Directorate of Blood Transfusion in Tehran Province.

### Quantitative real-time PCR (qRT-PCR)

Two milliliters (ml) of whole blood (WB) were collected in EDTA anticoagulant. For thalassemia patients, samples were obtained just prior to blood transfusion.

Total RNA was extracted from the WB using a commercial assay (Hybrid-R™ Blood RNA, GeneAll, Seoul, Korea) according to the manufacturer's instructions. The extracted RNA was assessed for its concentration and purity using a Nanodrop®, then stored at –70 °C. Several extracted RNAs were randomly electrophoresed on 1% agarose gel. The observation of 18s rRNA and 28s rRNA bands confirmed the accuracy of the RNA extraction process (Figure 2). cDNAs were synthesized by the random hexamer method (HyperScript™ first-strand synthesis kit, GeneAll, Seoul, Korea). PCR confirmed the quality of the synthesized cDNA.

Real-time PCR was performed using specific primers for the FAM132B gene, along with the HPRT (hypoxanthine-guanine phosphoribosyltransferase) gene as an internal control[13]. Each PCR reaction was carried out in a final volume of 20 µl, consisting of a mixture containing 10 µl of 2× SYBR Green-based

master mix, 0.4 µl of each primer (forward and reverse), 7.2 µl of DEPC-treated water, and 2 µl of cDNA. The amplification conditions for the PCR reactions were as follows: initial denaturation at 95 °C for 15 minutes, followed by 40 cycles with 15 seconds at 95 °C and 35 seconds at 60 °C. Table 1 represents the sequence of the primers used in this study.

All qRT-PCR analyses were performed in duplicate to ensure technical reproducibility. Relative gene expression levels of the FAM132B gene were calculated using the $2^{-\Delta\Delta CT}$ method, with CT values normalized to the reference gene (HPRT) and compared to those of the control group.

### Statistical analysis

The statistical computations and analyses were performed using GraphPad Prism software, version 8.2.0 (GraphPad Software Inc., CA, USA). Qualitative data were expressed as numbers and percentages (%), while quantitative data were presented as the mean ± standard deviation (SD) and range. The normality of data distribution was assessed using the Shapiro-Wilk test. Given the normal distribution of data, the one-way analysis of variance (ANOVA) test was used to compare the variance changes between groups. A *p*-value less than 0.05 was considered to be statistically significant.

**Table 1:** List of real-time PCR primers used in this study

| Gene | Primer | Reference |
|---|---|---|
| HPRT-F | 5'-CAGCAGTGAGCTCTTCACCA-3' | [13] |
| HPRT-R | 5'-CAAGAACACGGAGGTCCACT-3' | |
| FAM132B-F | 5'-GCCCTGGCGTCGTGATTAGT-3' | [13] |
| FAM132B-R | 5'-AGCAAGACGTTCAGTCCTGTC-3' | |

HPRT: hypoxanthine-guanine phosphoribosyl transferase

## RESULTS

Overall, 40 β-thalassemia patients were recruited for this study, comprising 22 males (55%) and 18 females (45%), with ages ranging from 21 to 66 years. The serum ferritin level of 50% of patients, including 8 (40%) BTI and 12 (60%) BTM patients, was more than 1000 ng/ml, which was considered iron overload according to the TIF guideline[24]. No statistically significant differences were observed in gender, age, hemoglobin value, and serum ferritin level between patients with BTM and BTI (Table 2).

The expression of the FAM132B gene was upregulated in patients with BTI compared to those with BTM ($p = 0.0151$). Despite the higher mRNA fold change in the BTI patients, we found no significant difference in the expression of the FAM132B gene between the beta-thalassemia patients (major and intermedia) and the control group (Table 3 and Figure 1).

**Table 2:** Demographic and laboratory data of β-thalassemia patients referred to the Zafar Adult Thalassemia Clinic of Tehran, Tehran Province, Iran

| Variable | Beta-thalassemia patients (N=40) | | |
|---|---|---|---|
| | Major (N=20) | Intermedia (N=20) | *p* |
| Gender | | | |
| Male | 13 (65%) | 9 (45%) | 0.21 |
| Female | 7 (35%) | 11 (55%) | 0.21 |
| Age (year) | | | |
| Mean ± SD | 34.95 ± 8.79 | 37.11 ± 12.26 | 0.54 |
| Range | 21-61 | 23-66 | |
| Hb (g/dl) | | | |
| Mean ± SD | 8.97 ± 1.03 | 8.37 ± 1.51 | 0.15 |
| Range | 7.1-10.5 | 5.8-10.4 | |
| Ferritin (ng/ml) | | | |
| Mean ± SD | 1713 ± 1401 | 1616 ± 1296 | 0.83 |
| Range | 222-6245 | 331-3925 | |

SD: standard deviation, Hb: hemoglobin. A *p*-value less than 0.05 was considered to be statistically significant.

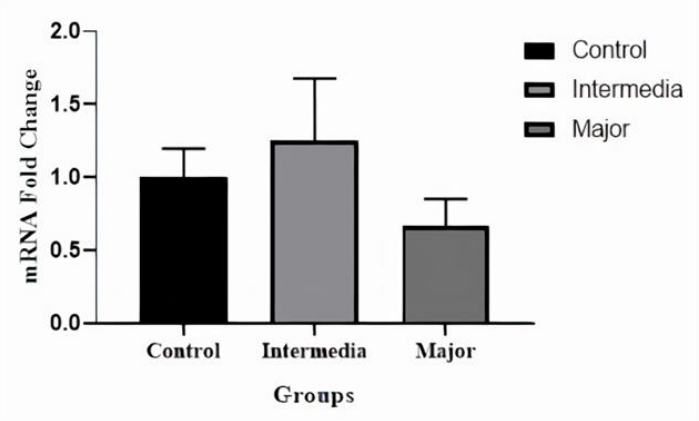


Figure 1. Comparison of the mRNA fold change (expression) of the FAM132B gene in the groups studied in the current research, including beta-thalassemia major and intermedia patients and the control group. The expression of the FAM132B gene was upregulated in patients with beta-thalassemia intermedia ($p = 0.0151$). Data are presented as mean ± SD (standard deviation).

**Table 3:** Comparison of the FAM132B gene expression in beta-thalassemia patients and the control group studied in the current research

| Groups Studied | Mean diff. | Fold change | 95.00% CI of diff. | Individual *p*-value |
|---|---|---|---|---|
| BTM vs control | -0.5861 | 0.666141 | -1.369 to 0.1965 | 0.1390 |
| BTI vs control | 0.3262 | 1.253707 | -0.4481 to 1.100 | 0.4021 |
| BTM vs BTI | -0.9123 | 0.531337 | -1.641 to -0.1833 | 0.0151 |

CI: confidence interval, BTM: beta-thalassemia major, BTI: beta-thalassemia intermedia. A *p*-value less than 0.05 was considered to be statistically significant.

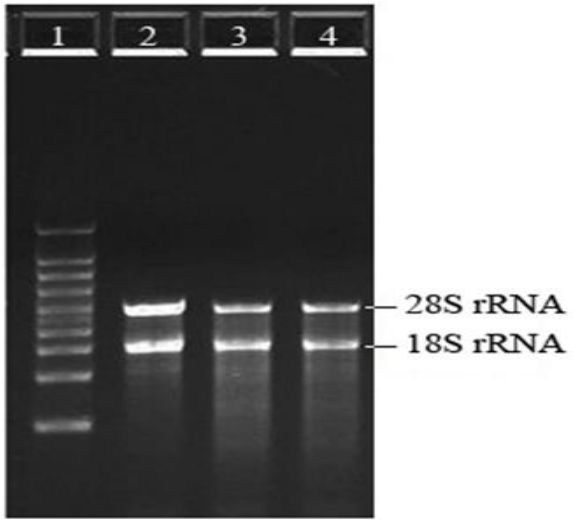


Figure 2. 18S rRNA and 28S rRNA electrophoresis bands related to RNAs extracted on 1% agarose gel. Lane 1: 100 bp ladder marker; lanes 2-4: RNAs extracted in this study

## DISCUSSION

In this study, we analyzed the expression of the FAM132B gene in beta-thalassemia patients using a real-time PCR assay. Our findings revealed a significant difference in the expression of the FAM132B gene between patients with BTM and those with BTI. The FAM132B gene was upregulated in BTI patients. To explain this difference, we postulated that since beta-thalassemia intermedia patients do not receive regular blood transfusions, erythropoietin (EPO) production is higher than in patients with beta-thalassemia major, compensating for the anemia[25]. The increased EPO secretion enhances FAM132B gene expression, as indicated by the results of studies performed[13,19,26]. On the other hand, regular blood transfusions in beta thalassemia major patients lead to a reduction in erythropoietic stimulation in these patients[27].

The ERFE is produced in response to EPO, blood loss, and ineffective erythropoiesis, leading to hepcidin suppression, which increases iron absorption and the release of iron from body resources[26]. Under physiological conditions, this process helps provide enough iron for hemoglobin synthesis and recovery from anemia. However, in pathological circumstances, including thalassemia and congenital dyserythropoietic anemia, the suppressive role of ERFE is associated with iron overload[28,29].

Through regular transfusion therapy, ineffective erythropoiesis is reduced in BTM; however, in BTI patients, this defective cycle leads to the production of more precursors, resulting in increased secretion of ERFE. Since blood transfusions in BTM patients are significantly more frequent than in BTI patients, it is expected that the levels of ferritin in BTM patients will be higher than in BTI patients. Blood transfusion is the main reason for iron loading in beta-thalassemia patients. However, iron overload also occurs in patients with BTI[30]. This is consistent with the serum ferritin level of patients in our study (Table 2). It is assumed that the underlying mechanism is a higher expression of erythroferrone in BTI patients. With the increase in available ERFE, the demand for iron also increases, which in turn enhances intestinal

absorption and allows for the production of more red blood cells.

In our study, despite the higher mRNA fold change in the BTI patients, no statistically significant difference was observed in the FAM132B gene expression between beta-thalassemia patients (major and intermedia) and the control group (Table 3 and Figure 1). In addition to its physiological role in normal erythropoiesis in healthy subjects, erythroferrone also plays a pathological role in blood disorders with ineffective erythropoiesis (IE), such as beta-thalassemias, where excessive production of erythroferrone following high levels of EPO ultimately leads to iron overload[13,31]. This may explain the statistically insignificant difference observed between the patients and the control group.

Most patients receive various regimens of chelation therapy, which affects their iron status. Genetics also plays a crucial role in iron homeostasis. Several single-nucleotide polymorphisms (SNPs) have been identified in the hepcidin gene[32,33], which modulates transcription, as may be the case with the ERFE. Thus, analyzing a large number of samples is necessary to offset the effect of these intervening variables. The ERFE is primarily produced in the bone marrow by erythroid precursors, particularly polychromatic erythroblasts. While the bone marrow provides the most suitable source for assessing the expression of the gene encoding ERFE, it is not easily accessible in patients[19]. Consequently, we decided to use whole blood for RNA extraction to assess gene expression. This approach, however, may introduce variability that could explain differences among study results.

## CONCLUSION

The expression of the FAM132B gene was different between beta-thalassemia major and intermedia patients. Our findings contribute to the understanding of iron regulation and highlight the need for further research with larger and more diverse sample sets to better elucidate the role of erythroferrone in iron homeostasis and as a potential therapeutic target in beta-thalassemia patients.

**Ethics approvals and consent to participate**

The Ethics Committee of Research approved this study in the High Institute for Research and Education in Transfusion Medicine (IR.TMI.REC.1396.037), Tehran, Iran. All participants signed informed consent.

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## CONFLICT OF INTEREST

The authors declare that they have no competing interests.

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