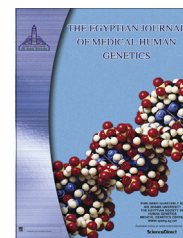




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ORIGINAL ARTICLE

# Study of the effect of HFE gene mutations on iron overload in Egyptian thalassemia patients



Manal Michel Wilson <sup>a</sup>, Hanan Al-Wakeel <sup>a</sup>, Fadwa Said <sup>a,\*</sup>,  
Mona El-Ghamrawy <sup>b</sup>, Mary Assaad <sup>c</sup>, Amal El-Beshlawy <sup>b</sup>

<sup>a</sup> Department of Clinical Pathology, Cairo University, Cairo, Egypt

<sup>b</sup> Department of Pediatrics, Faculty of Medicine, Cairo University, Cairo, Egypt

<sup>c</sup> Egyptian National Scientific and Technical information network, Egypt

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## KEYWORDS

HFE mutations;  
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**Abstract** *Background:* HFE gene mutations have been shown to be responsible for hereditary hemochromatosis. Their effect on iron load in  $\beta$ -thalassemia patients and carriers remains controversial.

*Objectives:* We aimed to determine the prevalence of HFE gene mutations (C282Y and H63D) in  $\beta$ -thalassemia patients and carriers and to investigate its effect on their serum ferritin levels.

*Patients and methods:* A total of 100  $\beta$ -thalassemia subjects; 75 patients and 25 carriers were screened for HFE gene mutations by PCR-RFLP. Serum ferritin measured by ELISA was evaluated in relation to HFE mutations.

*Results:* Twenty-eight  $\beta$ -thalassemia patients (37.3%) were heterozygotes for H63D mutation (H/D), 8 (10.7%) were D/D and 39 (52%) were negative (H/H). Among carriers, 4 (16%) were D/D and 21 (84%) were H/H homozygotes. C282Y mutant allele was not detected in any of the subjects. Serum ferritin levels were significantly higher in  $\beta$ -thalassemia patients heterozygotes or homozygotes for H63D mutation compared to those without mutation ( $p = 0.000$ ). Carriers homozygotes for H63D mutation showed significantly higher serum ferritin levels compared to those without mutation ( $p < 0.001$ ).

*Conclusion:* Homozygosity for H63D mutation tends to be associated with higher ferritin levels in beta-thalassemia patients and carriers suggesting its modulating effect on iron load in these cases.

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\* Corresponding author at: Department of Clinical and Chemical Pathology, Cairo University, Cairo 70050, Egypt. Tel.: +20 1000504190.

E-mail addresses: [mmw@tedata.net.eg](mailto:mmw@tedata.net.eg) (M.M. Wilson), [Hananwakeel@hotmail.com](mailto:Hananwakeel@hotmail.com) (H. Al-Wakeel), [f2said@yahoo.com](mailto:f2said@yahoo.com) (F. Said), [Mona.ghamrawy@kasralainy.edu.eg](mailto:Mona.ghamrawy@kasralainy.edu.eg) (M. El-Ghamrawy), [assaadmary@gmail.com](mailto:assaadmary@gmail.com) (M. Assaad), [amalelbeshlawy@yahoo.com](mailto:amalelbeshlawy@yahoo.com) (A. El-Beshlawy).

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## 1. Introduction

$\beta$ -Thalassemia constitutes a major public health problem in Mediterranean countries [1]. In Egypt, it is the most common chronic hemolytic anemia (85.1%) with a carrier rate of 5.3–9% and annual birth of 1000/1.5 million live births [2].

$\beta$ -Thalassemia is associated with ineffective erythropoiesis and ultimately iron overload [3], the later being the principal and multifaceted complication of  $\beta$ -thalassemia. Physiologically, iron overload is caused by increased iron absorption from the gastrointestinal tract as a consequence of ineffective erythropoiesis, and is greatly aggravated by chronic transfusion therapy. Malfunctions of the hepcidin–ferroportin axis contribute to the etiology of iron overload seen in thalassemia [4]. A remarkable variability of tissue iron distribution has been observed in  $\beta$ -thalassemia with liver, heart and endocrine glands being the organs most severely affected [5]. Death from cardiac failure is the most common clinical consequence of iron excess. Therefore, effective iron chelation therapy is a critical component in the management of thalassemia [5].

Hereditary hemochromatosis, an autosomal recessive condition involving iron metabolism, results in multiorgan toxic iron overload [6]. Mutations in the HFE gene have been reported to be responsible for primary hemochromatosis and may play a role in the severity of secondary hemochromatosis [7]. One of the commonest mutations in the HFE gene involves a C to G substitution at nucleotide 187 that changes amino acid 63 from histidine to aspartic acid (H63D). This mutation has been reported to reduce the affinity between transferrin receptor and iron transferrin [8]. Another common mutation includes the cysteine-to-tyrosine substitution at amino acid 282 in the HFE gene (C282Y mutation) [9].

The effect of these mutations on iron load in  $\beta$ -thalassemia patients and carriers remains controversial. Interaction between  $\beta$ -thalassemia and HFE gene mutations may increase the likelihood of developing iron overload in thalassemic patients [10]. Thus the impact of these mutations analysis on the management of iron overload could be significant, allowing early accurate diagnosis and proper management to overcome complications of secondary hemochromatosis [6].

This study aimed to determine the prevalence of HFE gene mutations (C282Y and H63D) among  $\beta$ -thalassemia patients and carriers and to investigate the effect of these mutations on their serum ferritin levels.

## 2. Subjects and methods

In this cross-sectional study, a total of 100  $\beta$ -thalassemia subjects; 75  $\beta$ -thalassemia patients (homozygous or compound heterozygous) and 25 carriers were screened for HFE gene mutations (H63D and C282Y) by polymerase chain reaction-restriction fragment length polymorphism method (PCR-RFLP). Serum ferritin was measured for all subjects by Microparticle Enzyme Immunoassay (AxSYM, Abbott, USA) at diagnosis and  $\beta$ -globin gene mutations were also determined by reverse hybridization technique. All  $\beta$ -thalassemia patients (45 males and 30 females, mean age  $3.2 \pm 2.7$  years) were diagnosed and followed at the Pediatric Hematology Clinic of Cairo University. Diagnosis of  $\beta$ -thalassemia was based on clinical presentation, hematological indices, high performance liquid chromatography (HPLC) [using the VARIANT II Beta-Thalassemia Short Program, Bio-Rad Laboratories].

Their baseline serum ferritin at diagnosis was evaluated in relation to the HFE mutations. Twenty-five heterozygotes for  $\beta$ -thalassemia (12 males and 13 females, mean age  $28.3 \pm 3.9$  years) attending for genetic screening or parents

or sibs of our patients were enrolled as carriers. All enrolled subjects had no evidence of active liver disease or recent or present infections or inflammatory illnesses that might affect ferritin levels. All subjects or guardians gave informed consent before enrollment. The study has been carried out in accordance with The Code of Ethics of The World Medical Association (Declaration of Helsinki) for experiments in humans. The study protocol was approved by the Ethics Committee of the Cairo University.

### 2.1. Molecular testing

DNA was first extracted from peripheral blood samples of the affected children and their parents using QiAmp DNA blood minikit (Catalogue No. 51104). Mutations were identified by the reverse dot blot hybridization technique (RDB). For RDB, a panel of probes ( $n = 22$ ) using the beta globin strip assay was used ( $\beta$ -Globin StripAssay MED kit, VIENNA LAB, 116211, Austria) [11].

### 2.2. Detection of HFE gene mutations

Two primer sets were used for DNA amplification, the first, 5'-TGGCAAGGG'TAAACAGATCC-3 and 5'-CTCAGGCAC-TCCTCTCAACC-3 for the H63D mutation site and the second, 5'-ACATGGTTAAGGCCTGTTGC3' and 5'-GCCA CATCTGGCTTGAAATT-3' for the C282Y mutation site [9]. PCR was performed using a Perkin Elmer Thermal Cycler Gen Amp 9600 (Applied Biosystems, UK). A total volume of 25  $\mu$ l PCR reaction containing 12.5  $\mu$ l of ready-to-use PCR Master Mix supplied by *QIAGEN*<sup>®</sup>, Cat. No: 201443, 5.5  $\mu$ l of Nuclease-free water, 1  $\mu$ l (20 pmol) of primer F, 1  $\mu$ l (20 pmol) of primer R and 5  $\mu$ l (100 ng) of genomic extracted DNA.

PCR cycles were 35 cycles of 96 °C for 2 min, 96 °C for 30 s, 56 °C for 1 min, 72 °C for 1 min and final extension step of 72 °C for 5 min. Ten micro liters of the PCR products were subsequently digested with the restriction enzymes SnaBI Enzyme (*Fast Digest*<sup>®</sup> SnaBI) (C282Y mutation) supplied from Fermentas Life Sciences, USA Reagents Code No. #FD1794 and BclI (H63D mutation) supplied by New England Biolab reagent code No.: R0160S. The digestion products were electrophoresed on a 3% gel (1:1 agarose/Nusiev). Amplification with the primers for codon 282 produced a 400 bp fragment in the wild genotype, in the mutant genotype it would produce 2 fragments of 290 bp and 110 bp after endonuclease digestion. On the other hand, the wild genotype of H63D mutation gives a single band at 187 bp, the heterozygous H/D genotype gives 2 bands at 187 and 209 bp and the homozygous mutant D/D genotype gives a single band at 209 bp [9]. DNA size marker (100 pb) was used (Thermo Scientific GeneRuler 100 bp Plus DNA Ladder, ready-to-use #SM0323).

### 2.3. Statistical methods

Data were coded and entered using statistical package SPSS version 17. Data were expressed as mean  $\pm$  standard deviation ( $\pm$ SD), median, range, frequencies and percentages when appropriate. Numerical variables were compared using Student *t* test for independent samples when normally distributed and Mann Whitney *U* test when not normally distributed. For

comparing categorical data, Chi square ( $\chi^2$ ) test was performed. One way ANOVA was used to test for differences between groups. Significance level was defined as  $p < 0.05$ .

### 3. Results

$\beta$ -Thalassemia patients showed significantly lower mean hemoglobin levels and higher median serum ferritin compared to thalassemia carriers ( $7.9 \pm 1.6$  g/dl vs.  $9.9 \pm 2.3$  g/dl;  $p$  value  $< 0.001$  and  $386$  ng/ml vs.  $216$  ng/ml;  $p = 0.03$  respectively). The most prevalent underlying genetic mutations of  $\beta$  globin gene in the  $\beta$ -thalassemia patients were IVS 1.110 (55%) followed by the IVS 1.6 (36%).

#### 3.1. Prevalence of HFE gene mutations

Twenty-eight of 75  $\beta$ -thalassemia patients (37.3%) were heterozygotes for the H63D mutation (H/D), 8 (10.7%) were D/D homozygotes and 39 (52%) were negative for the mutation (H/H homozygotes). Among carriers, 4 (16%) were D/D homozygotes and 21 (84%) were H/H homozygotes (Fig 1). The wild type (H/H genotype) presented at significantly higher frequency among carriers ( $p = 0.002$ ), whereas the mutant heterozygous genotype was significantly more prevalent among thalassemia patients ( $p = 0.002$ ) (Table 1). The C282Y mutant allele was not detected in either the patients or carriers.

#### 3.2. H63D gene polymorphisms and serum ferritin

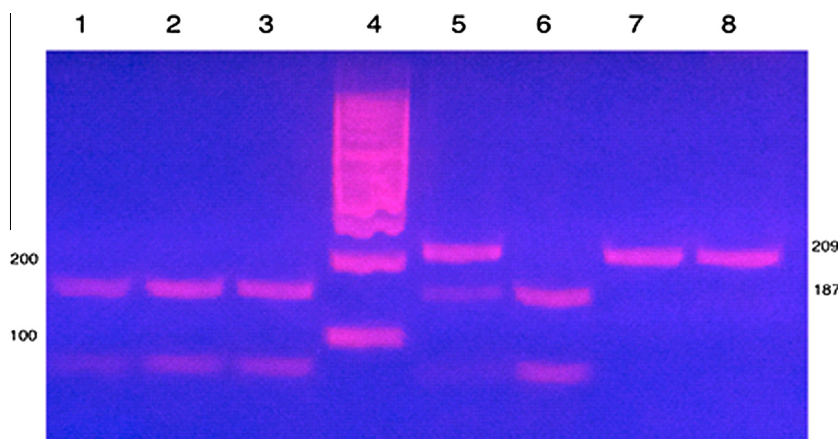
Serum ferritin levels were compared to H63D genotypes in  $\beta$ -thalassemia patients and carriers (Table 2). Serum ferritin levels were significantly higher in  $\beta$ -thalassemia patients who were heterozygotes or homozygotes for the H63D mutation compared to those without the mutation ( $p < 0.001$ ).  $\beta$ -Thalassemia carriers homozygotes for the H63D mutation showed significantly higher serum ferritin levels compared to those without the mutation ( $p < 0.001$ ).

#### 3.3. H63D and $\beta$ globin gene mutations

No statistically significant relation between the H63D gene mutation and genotype of the patients or the carriers ( $p > 0.05$ ).

### 4. Discussion

Co-inheritance of HFE gene mutations may have a substantial role in iron overload in  $\beta$ -thalassemia patients and carriers. Results from different studies are rather conflicting. We hereby report the prevalence of H63D mutations and the absence of C282Y mutations among a cohort of 100 Egyptian  $\beta$ -thalassemia patients and carriers. We also evaluated the effect of these mutations on their steady state baseline serum ferritin levels.



**Figure 1** PCR polymorphisms of H63D mutation of HFE gene in  $\beta$ -thalassemia patients. PCR of H63D mutation in HFE gene showing: Lane 4: Molecular weight marker 100 bp. Lanes 1, 2, 3 and 6: Shows the H/H wild genotype with a single band at 187 bp. Lane 5: Shows the H/D heterozygous genotype with 2 bands at 187 and 209 bp respectively. Lanes 7 and 8: Shows the D/D homozygous mutant genotype with a single band at 209 bp.

**Table 1** Results of the PCR-RFLP demonstrating different patterns of polymorphism of HFE: H63D polymorphism.

	$\beta$ thalassemia patients $N = 75$	$\beta$ thalassemia carriers $N = 25$	$P$ value
Wild type (H/H genotype) Number (%)	39 (52%)	21 (84%)	0.002*
Heterozygous (H/D genotype) Number (%)	28 (37.3%)	0	0.002*
Homozygous (mutant) (D/D genotype) Number (%)	8 (10.7%)	4 (16%)	0.002*

\* High statistical significance.

**Table 2** Serum ferritin levels in  $\beta$ -thalassemia patients and carriers according to their H63D genotype.

H63D genotype	$\beta$ -Thalassemia patients ( $n = 75$ )			$\beta$ -Thalassemia carriers ( $n = 25$ )		
	No. of subjects (%)	Ferritin (ng/ml) Mean $\pm$ SD	<i>P</i> value	No. of subjects (%)	Ferritin (ng/ml) Mean $\pm$ SD	<i>P</i> value
H/H	39(52%)	297.2 $\pm$ 175.8	<0.001*	21(84%)	221.9 $\pm$ 142.9	<0.001*
H/D	28(37.3)	565.3 $\pm$ 358		–	–	
D/D	8(10.7%)	920.4 $\pm$ 508.2		4(16%)	969.8 $\pm$ 290.3	

\* *P*-values: H/H vs. H/D; H/D vs. D/D; H/H vs. D/D <0.001.

The prevalence of HFE gene mutations vary widely between different populations; being highly prevalent among European population [12], relatively less common in Brazil [13], Japan [14] and Thailand [15]. Available data among Egyptian population or thalassemia patients are rather limited. In this study, the reported frequency of HFE gene mutations was comparable to that in previous studies in Egyptian thalassemia patients [16,17] and non-Egyptians populations with different ethnic backgrounds whether thalassemia patients or not [12,18,19]. However, in our study homozygous mutant (D/D genotype) was detected among our thalassemia patients and carriers (10.7% and 16% respectively) a finding which was not reported in the previous Egyptian study [16], the number of studied patients can explain this difference. Heterozygous mutant genotype of H63D was more prevalent among our  $\beta$ -thalassemia patients compared to carriers, however a more extended study with higher number of carriers and patients would be needed to confirm this finding.

C282Y mutation was reported to be responsible for 60% of hereditary hemochromatosis cases in the Mediterranean population [20], with a North to South decrease in frequency [21,22]. In a Brazilian cohort, a high frequency of C282Y mutation among beta thalassemia patients suggested that it may worsen the clinical picture of these individuals. C282Y mutation was not detected in any of our patients or carriers and this is in agreement with other studies among Egyptian and non-Egyptian populations which reported the extreme rarity or even the absence of C282Y mutation [10,16,18,23–25]. This discordance may be attributed to differences in racial and ethnic backgrounds.

Despite advances in methods used to assess iron load in thalassemia patients, serum ferritin measurement remains to be the widely and most commonly used tool especially in developing countries. On comparing steady state serum ferritin levels with H63D genotypes in our cohort, both homozygote and heterozygote states showed significantly higher levels of serum ferritin among  $\beta$ -thalassemia patients.  $\beta$ -Thalassemia carriers with the homozygote mutant genotypes had significantly higher serum ferritin levels than those with no mutation. This may confirm the possible role of H63D mutation in increasing iron storage when in interaction with other genetic determinants [26]. Two independent pathways have been proposed to regulate iron metabolism, namely the erythroid regulator, which modulates intestinal iron absorption in response to the needs of the erythron, and the storage regulator, which controls iron accumulation [27–29]. Despite the hypothesis that the erythroid regulator (beta-thalassemia) seems to be more pronounced than the storage regulator (the defective HFE gene) in determining the degree of iron absorption [27,28], our results along with findings from other studies

[18,30–32] support the observation that coinheritance of  $\beta$ -thalassemia with heterozygosis or homozygosis for H63D are associated with increased iron load. However, results are still controversial [24,29,33,34].

The most prevalent underlying genetic mutations of  $\beta$  globin gene in our  $\beta$ -thalassemia patients and carriers were IVS 1.110 (55% and 44% respectively) followed by the IVS 1.6 (36% and 20% respectively). This goes in agreement with other studies including similar ethnic groups [34–38]. This study showed no correlation between these mutations and the H63D genotype.

In conclusion, homozygosity for the H63D mutation tends to be associated with higher ferritin levels in beta-thalassemia patients and carriers. This suggests that this mutation may have a modulating effect on iron load in these patients. Proper and timely management prevents the hazard of iron overload.

#### Conflict of interest

All authors have no conflict of interest that could inappropriately influence this work.

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