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# Analysis of G71D Mutation of HAMP Gene and H63D Mutation of HFE Gene in ß-Thalassemia Major Patients

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## ABSTRACT

***Background:*** Hepcidin encoded by HAMP gene plays a key role in modulating iron absorption in ß-thalassemia. Hepcidin deficiency due to either mutations in HFE gene encoding the hemochromatosis protein (HFE) or in the HAMP gene have been implicated in iron overload.

***Aim of the Work:*** To establish the presence of G71D mutation of HAMP gene and H63D mutation of HFE gene in ß-thalassemia major patients as well as to assess their impact on iron overload in these patients.

***Material and Methods:*** This study included 52 ß-thalassemia major patients and 46 age- and sex- matched healthy controls. Genotyping of G71D of HAMP and of H63D of HFE variants was performed by polymerase chain reaction-restriction fragment length polymorphism analysis. Estimation of iron overload was based on serum ferritin and transferrin saturation.

***Results:*** Among the studied ß-thalassemia patients, 30 (57.7%) carried the wild-type profile, 13 (25%) carried G71D mutation of HAMP gene, 12 (23.1%) carried the H63D mutation of HFE gene and 3 (5.8%) carried both mutations. Both HAMP-G71D and HFE-H63D mutations observed among patients were in the heterozygous condition. Patients with either HAMP-G71D or HFE-H63D variants did not show significant difference in iron overload parameters in relation to wild-type patients.

***Conclusion:*** The G71D mutation of HAMP gene and H63D mutation of HFE gene are common variants detected in about one fourth of the studied ß-thalassemia major patients. Neither the HAMP-G71D mutation nor the HFE-H63D mutation is a major determinant of iron overload in patients with ß-thalassemia major.

***Key Words:*** *ß-thalassemia major – HAMP – G71D – HFE – H63D.*

## INTRODUCTION

Hepcidin, encoded by HAMP gene, is a 25 amino acid peptide that, in addition to being involved in innate immunity **[1]**, appears to play a crucial role in iron homeostasis in humans, regulating both iron absorption from the intestine and its recycling by macrophages **[2-5]**. Hepcidin is down-regulated by erythropoiesis **[2]**, anemia, and hypoxia **[3]**, whereas it is up-regulated by iron overload **[4]** and inflammation **[3,5-7]**. Hepcidin deficiency due to either mutations in HFE gene encoding the hemochromatosis protein (HFE) or in the HAMP gene is the cause of iron overload in most forms of hereditary hemochromatosis (HH) **[8]**. Furthermore, hepcidin deficiency is the main or contributing factor of iron overload in iron-loading anemias such as ß-thalassemia **[9]**.

In ß-thalassemia major, transfusions rather than dietary iron absorption are the predominant cause of iron overload. In chronically transfused patients, hepcidin concentrations are significantly higher than in nontransfused patients, presumably due to both increased iron load and the alleviation of ineffective erythropoiesis. However, hepcidin concentrations decrease in the intervals between transfusions, as the effect of each transfusion wears off **[10-12]**. During those periods, decreased hepcidin and the resulting increase in intestinal iron absorption may be significant contributors to patients' iron load **[9]**.

A missense mutation in HAMP gene that leads to substitution of glycine 71 by aspartic acid (G71D) due to a G→A substitution at nucleotide 212 in exon 3 changes the charge of amino acid 71 and is likely to affect the activity of hepcidin **[13]**. H63D is a variant of the HFE

gene characterized by a G→C change at nucleotide 187 that results in a change in histidine at position 63 to aspartic acid. This mutation alters dietary iron absorption **[14]**. The interaction of the mutations of genes influencing iron homeostasis with thalassemias may have a synergistic effect, increasing the iron absorption and storage **[15,16]**. Knowledge of mutation prevalence of the genes influencing iron overload would ensure preventive treatment for iron overload. The aim of the present study was to establish the presence of G71D mutation of HAMP gene and H63D mutation of HFE gene in ß-thalassemia major patients as well as to assess their impact on iron overload in these patients.

## MATERIAL AND METHODS

*Subjects:*

This study included 52 ß-thalassemia major cases regularly attending the Hematology Clinic of the New Children Hospital, Cairo University and 46 healthy age- and sex-matched control subjects. Patients' age ranged from 2 to 25 years with a mean age of 12 years. They were 23 (44.2%) males and 29 (55.8%) females. The age of the control subjects ranged between 2 and 23 years with a mean age of 9.4 years. They were 28 males and 18 females. The diagnosis of ß-thalassemia was based on clinical presentation, hematological indices, iron overload and hemoglobin electrophoresis. For each subject of patients and controls, 4mL peripheral venous blood was collected in EDTA vials for molecular studies, and 2mL of blood without anticoagulant was collected for evaluation of iron overload parameters. An informed consent approved by the institutional Ethics Committee was obtained from all participants or their parents.

*Clinical parameters:*

All patients were on regular blood transfusion; 19/52 (36.5%) patients received 50 or less transfusions per life (ranging from 12 to 49 and median of 25) while 33/52 (63.5%) patients received more than 50 transfusions in life (ranging from 52 to 294 and median of 120).

The studied patients experienced thalassemia-related complications in the form of skull deformities and mongoloid facies in 45/52 (86.5%) and bony aches in 30/52 (57.6%). Hepatitis C virus infection was detected in 16/52 (30.8%) patients and hepatitis B virus infection in 1/52 (1.9%) patient. No cardiovascular or endocrinal complications were reported in any of the studied patients.

All patients were on iron chelation therapy: 26/52 (50%) patients received oral deferiprone alone, 24/52 (46%) patients were on S.C. desferrioxamine therapy and 2/52 (4%) patients received combined desferrioxamine and deferiprone treatment. Compliance to iron chelation therapy was verified in 25/52(48%) of chelated patients whereas 27/52 (52%) received irregular iron chelation.

*Iron overload parameters:*

Iron profile was assessed for both ß–thalassemia patients and controls. Serum iron and total iron binding capacity (TIBC) were measured colorimetrically and serum transferrin saturation was calculated. Serum ferritin was determined by Microparticle Enzyme Immunoassay (AxSYM, Abbott, USA) after an overnight fast.

*Genotypic analysis:*

Genomic DNA was extracted from peripheral blood leukocytes by QIAamp DNA Blood Mini Kit (#51104, QIAGEN). Genotyping of G71D of HAMP and of H63D of HFE variants was performed by polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) analysis according to Merryweather-Clarke et al. **[13]** and Feder et al. **[17]**, respectively. For each individual, we systematically amplified two PCR fragments surrounding both mutations in two separate reactions, using the following pairs of primers: for G71D mutation; sense primer 5'-ATGCAGGGAGGTGTGTTA GGAGGCT- 3' and antisense primer 5'-TGCAAGGC-AGGGTCAGGACAAGCTCTT AGC- 3', for H63D mutation; sense primer 5'-ACATGGTTAA-GGCCTGTTGC-3' and antisense primer 5'-GCCACATCTGGCTTG AAATT-3'. PCR was performed in reactions containing 3µL of extracted DNA in the presence of 1µL of each primer (10 pmole/µL), 12.5µL of Taq PCR Master Mix (QIAGEN) and 7.5µL nuclease-free water in a total volume of 25µL. The PCR conditions consisted of an initial melting temperature of 94°C for 5 minutes followed by 30 cycles of denaturation at 94°C for 30 seconds, annealing at 56°C for 30 seconds and extension at 72°C for 1 minute. A final extension step of 10 minutes at 72°C terminated the reaction.

The sizes of the amplified fragments were 714bp for G71D (exons 2 and 3) and 207bp for H63D (exon 2). Ten microliters of the amplified products was subjected to separate digestion with 10 units of Aci I (FastDigest® AciI, #FD1794 - Fermentas Life Sciences) for the G71D mutation at 37°C for 15 minutes, as well with10 units of Bcl I (#R0160S - New England Biolabs) for the H63D substitution at 50°C for 1 hour, according to the manufacturer's recommendations. The digested products were then run on a 2.5% agarose gel for 1 hour and photographed under UV light (Fig. 1).

*Statistical methods:*

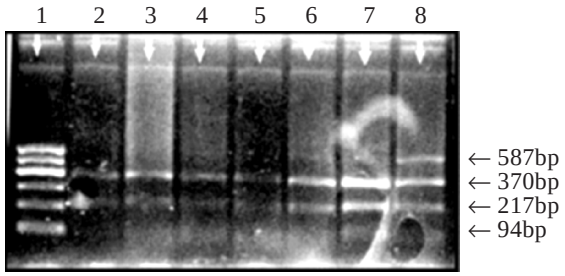
Statistical calculations were done using computer programs SPSS (Statistical Package for the Social Science; SPSS Inc., Chicago, IL, USA) version 15 for Microsoft Windows. Comparison of numerical variables was done using Student *t*-test in comparing 2 groups when normally distributed and Mann Whitney U test when not normally distributed. For comparing categorical data, Chi square ($\chi^2$) test was performed. Multivariate analysis model was used to test for the preferential effect of important variable(s) on ferritin level. *p*-values less than 0.05 was considered statistically significant **[18]**.

## RESULTS

Among the studied ß-thalassemia patients, 30 (57.7%) carried the wild-type profile, 13 (25%) carried G71D mutation of HAMP gene, 12 (23.1%) carried the H63D mutation of HFE gene and 3 (5.8%) carried both mutations. Both HAMP-G71D and HFE-H63D mutations observed among patients were in the heterozygous condition. The allelic frequency of G71D and H63D variants among the studied patients were 12.5% and 11.5%, respectively (Table 1).

Of the healthy controls studied, 37/46 (80.4%) carried a wild-type genetic profile in both genes, 6/46 (13.0%) had a variation in HAMP-G71D, 4/46 (8.7%) in HFE-H63D, and 1/46 (2.2%) in both. Both HAMP-G71D and HFE-H63D mutations observed in the control group were in the heterozygous condition. The allelic frequency of G71D and H63D variants among controls were 6.5% and 4.3%, respectively. Compared to controls, ß-thalassemia patients showed borderline significant higher frequency of H63D mutation (23.1% vs. 8.7%, $p$=0.062). No statistically significant difference in gene frequencies of G71D mutation was observed between studied patients and controls (Table 1).

A: G71D mutation




B: H63D mutation

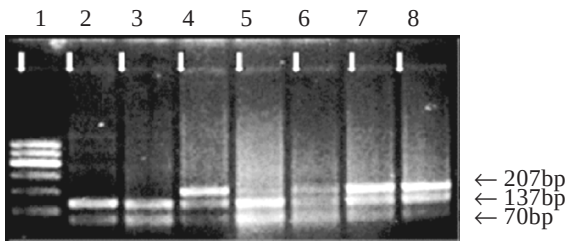



Fig. (1): Agarose gel electrophoresis of PCR fragments digested by restriction enzymes. (A) Diagnosis of G71D mutation: Aci I digestion of a 714bp PCR product containing HAMP exons 2 and 3. Wild-type digestion product sizes are 370, 217, 94 and 33bp; digestion products from the mutant allele are 587, 94 and 33bp. Lane 1: PCR marker of 100bp, Lanes 2, 3, 4, 5, 6, 7: Wild type individuals, Lane 8: heterozygous individual. (B) Diagnosis of H63D mutation: Bcl I digestion of a 207bp PCR product containing HFE exon 2. Wild-type digestion product sizes are 137 and 70bp. The mutation abolishes the restriction site. Lane 1: PCR marker of 100bp, Lanes 2, 3, 5 wild type individuals, Lanes 4, 6, 7, 8 heterozygous individuals.

Table (1): Comparison of genotype and allele frequencies of G71D and H63D mutations between ß-thalassemia patients and controls.

| Mutation | No. / Genotype frequency (%) | | *p* value | Allele frequency (%) |
|---|---|---|---|---|
| | Wild-type | Heterozygous | | |
| *G71D:* | | | | |
| Thalassemic patients (n=52) | 39 (75.0) | 13 (25.0) | 0.200 | 12.5 |
| Controls (n=46) | 40 (86.0) | 6 (13.0) | | 6.5 |
| *H63D:* | | | | |
| Thalassemic patients (n=52) | 40 (76.9) | 12 (23.1) | 0.062 | 11.5 |
| Controls (n=46) | 42 (91.3) | 4 (8.7) | | 4.3 |

$p<0.05$ is statistically significant.

Patients carrying either HAMP-G71D or HFE-H63D variants did not show statistically significant difference in iron overload parameters in relation to wild-type patients (Table 2).

Table (2): Comparison of iron profile parameters between wild-type patients and patients with HAMP-G71D variant or patients with HFE-H63D variant.

| ß-Thalassemia patients | Transferrin saturation (%) | Serum ferritin (ng/mL) |
|---|---|---|
| *G71D variant:* | | |
| Wild-type patients (n=39) | 79.3±18.7 | 2406.7±1214.5 |
| Heterozygous patients (n=13) | 77.8±20.9 ($p$=0.775) | 2376.8±1165.4 ($p$=1.00) |
| *H63D variant:* | | |
| Wild-type patients (n=40) | 78.7±19.7 | 2295.5±1260.8 |
| Heterozygous patients (n=12) | 79.9±17.6 ($p$=0.853) | 2744.8±878.6 ($p$=0.140) |

Statistical analyses are all related to the wild-type group, $p<0.05$ is statistically significant.

Multivariate regression analysis was done to reveal the independent association of factors that may significantly affect serum ferritin level among the study sample. It included age of onset of disease, number of transfusions in life, compliance to iron chelation therapy, presence of HCV antibody, presence of G71D mutation of HAMP gene, presence of H63D mutation of HFE gene and number of mutations harbored by the studied patients. Analysis revealed that only the number of blood transfusions per life significantly increases serum ferritin level ($p$=0.003).

Table (3): Multivariate regression analysis of independent factors that may affect serum ferritin level among the studied patients.

| Variable | Coefficient | $p$-value | 95% Confidence Interval | |
|---|---|---|---|---|
| | | | Upper limit | Lower limit |
| Age at onset | 469.200 | 0.149 | –174.433 | 1,112.834 |
| No. of Transfusions/life | 7.753 | 0.003* | 2.864 | 12.643 |
| Compliance to chelation | 184.672 | 0.655 | –641.102 | 1,010.446 |
| HCV Ab | 436.714 | 0.395 | –587.958 | 1,461.386 |
| H63D of HFE | 400.658 | 0.570 | –1,009.060 | 1,810.375 |
| No. of mutations | 502.243 | 0.286 | –433.792 | 1,438.279 |

*$p$-value <0.05 is statistically significant.

## DISCUSSION

In the present study, we analyzed the frequency of G71D mutation of HAMP gene and H63D mutation of HFE gene in 52 TM patients and 46 control subjects. Both mutations were found in about one fourth of the studied TM patients in a heterozygous condition. Their allelic frequencies were 12.5% and 11.5% for G71D and H63D variants, respectively. A lower frequency of both mutations was observed among control group (13.0% and 8.7% for G71D and H63D, respectively) with a borderline statistically significant difference regarding H63D mutation compared to the studied patients ($p$=0.062).

The frequency of G71D mutation of HAMP gene detected in the present study is higher than that reported by earlier studies. G71D mutation of HAMP gene was detected in the general north European population at an allele frequency of 0.3% **[13]** and has been identified in France **[19]**, Italy **[20]** and UK **[13]**. The H63D mutation has a prevalence of approximately 16% in the European population **[17,21]**. In accordance to our results, earlier Egyptian studies reported that the allele frequency of H63D mutation ranged from 13 to 30% in thalassemic patients and between 10 and 11% in controls **[22,23]**.

In the current study, the presence of either G71D mutation of HAMP gene or H63D mutation of HFE gene did not seem to influence iron overload in ß-thalassemia major patients. The functional relevance of the G71D amino acid substitution is not clear. However, it must be emphasized that this missense mutation is lo-

cated between 4 of the 8 structural cysteines of the 25-amino acid mature hepcidin peptide, and that the change of a neutral amino acid to an acidic residue frequently leads to crucial protein structure modifications **[19]**. Conflicting results were reported regarding the impact of this mutation as a possible modifier in iron overload diseases **[13,19,20,24]**.

It has been reported that the H63D mutation has an impact on iron overload in patients with beta thalassemia trait and thalassemia major **[25-27]**. Nevertheless, among the studied TM patients, no statistically significant difference in iron overload parameters was found between patients carrying HFE-H63D variant and wild-type patients. In literature, the significance of H63D was uncertain. H63D homozygosity has been found in association with an iron overload genotype and a study in mice showed that H63D mutation altered the normal HFE pathway, increasing iron overload **[28]**. Our data are in agreement with different groups who reported that the presence of H63D heterozygous state does not influence iron overload in ß-thalassemia major or minor **[29,30]**. In contrast, when the H63D mutation is found in the homozygote state, it may influence the ferritin levels of ß-thalassemia carriers **[31]**.

Multivariate regression analysis of independent factors that may significantly affect serum ferritin level was done in the present study and revealed that only the number of blood transfusions taken in life significantly increased serum ferritin level ($p$=0.003). Iron overload is an inevitable consequence of regular blood transfusion and can be seen after only 10-20 transfusions in patients with thalassemia **[32]**. The number of mutations harbored by the studied patients whether single or double mutations did not affect serum ferritin level in the multivariate regression model ($p$=0.286). This contrasts the hypothesis proposed by Duca and colleagues who suggested that the iron burden could be aggravated by the co-existence of mutations in HFE and HAMP genes in ß-thalassemia major patients poorly responsive to chelation therapy **[33]**.

Serum ferritin evaluation is a simple and convenient approach for assessment of iron overload that is widely used in clinical practice. Limitation of our study was inability to assess liver iron concentration (LIC) which remains the reference standard for determining iron load in patients at risk of iron overload. Many studies have assessed the correlation between serum ferritin levels and LIC, demonstrating a good correlation in patients with thalassemia major, mainly at lower LIC values **[34,35]**.

*Conclusion:*

The G71D mutation of HAMP gene and H63D mutation of HFE gene are common among ß-thalassemia major patients. Neither the HAMP-G71D mutation nor the HFE-H63D mutation is a major determinant of total body iron status in patients with ß-thalassemia major. The frequent occurrence of ß-thalassemia major and HAMP-G71D and HFE-H63D gene mutations raises the possibility of genetic interactions and emphasizes the value of screening for other HAMP and/or HFE mutations in thalassemias to modify treatment modalities of iron overload.

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