Haemochromatosis gene mutation H63D is a risk factor for iron overload in Egyptian beta- thalassemic children

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ABSTRACT

Introduction: Iron overload is the main cause of morbidity and mortality in patients with β-thalassemia.

The Aim: The aim of this study was to evaluate the prevalence of genetic markers (HFE mutations C282Y and H63D) among Egyptian β-thalassemic Children and its effect on their iron status.

Patients and Methods: 59 β-thalassemic children attending the pediatric hematology clinic in Menoufiya University Hospital (23 thalassemia major, 23 thalassemia intermedia and13 thalassemia trait) with 50 apparently healthy, Egyptian children (control group) were screened for the prevalence of these two mutations by digestion of PCR products (RFLP). Serum ferritin level was measured by ELISA.

Results: Neither carrier status for the C282Y allele nor homozygous status for the H63D allele were detected in any of the thalassemic children or the 50 controls. The H63D heterozygous state was detected in 15 (25.4%) thalassemic patients with an allele frequency of 12.71% and in 11 (22%) controls with an allele frequency of 11%. with no significant difference between the thalassemic groups and the controls. The prevalence of carriers for the H63D mutation was 26.1% with an allele frequency of 13.04% in patients with either β- thalassemia major or intermedia, while in β- thalassemia trait the prevalence of this mutation was 23.1% with an allele frequency of 11.54%. There were significant higher levels of the mean yearly serum ferritin in both β-thalassemia major and intermedia patients who are heterozygotes for the H63D mutation compared to those without this mutation. The mean serum ferritin levels were positively correlated with the age of the patients. On the other hand, the prevalence of iron -induced complications was not statistically different between patients carrying or not carrying this mutation (among TM and TI).

Conclusions: There is no difference in the prevalence of H63D mutation between β-thalassemic patients and the normal children and the presence of a heterozygous H63D status and older age are two risk factors for iron overload in Egyptian β-thalassemic children.

Abbreviations: RFLP= Restriction Fragment Length Polymorphism, HCV=Hepatitis C Virus, ALT = Alanine aminotransferase, AST =Aspartate aminotransferase

Key Words:

Thalassemia, iron overload, hereditary hemochromatosis, HFE gene.

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INTRODUCTION

The thalassemias are the commonest monogenic disorders in the world and globally it is estimated that there are 270 million carriers, of which 80 million are carriers of β-thalassemia.[1]

β- Thalassemia is an autosomal recessive disorder of hemoglobin synthesis. It is caused by a direct down-regulation in the synthesis of structurally normal β chains. Due to the excess of α-globin chains relative to β-globin chains; α-globin tetramers (α4) are formed, and these inclusions interact with the red cell membrane and shorten red cell survival, leading to anemia and increased erythroid production.[2]

The clinical manifestations of β- thalassemia are extremely diverse, spanning a broad spectrum from the transfusion-dependant state of thalassemia major to asymptomatic state of thalassemia trait. Between these two clinical extremes lies the clinical syndrome of thalassemia intermedia which comprises a diverse spectrum of phenotypes from a condition that is slightly less severe than transfusion dependence to one that is asymptomatic and often identified through a routine blood test[1]. A common complication of β-thalassemia involves organ damage from iron overload (hemosidrosis) which is not only an inevitable consequence of prolonged transfusion therapy[3] but also depends on the increased intestinal iron absorption which is proportional to the degree of erythroid hyperplasia.[4]

The major determinant of the clinical severity in thalassemia syndromes is the underlying mutations of the beta-globin gene and the degree of iron overload. Patients who inherit two β-thalassemia (severe) mutations usually have β-thalassemia major (TM), a severe anemia requiring life-long treatment with blood transfusions to maintain satisfactory levels of hemoglobin (Hb) and iron-chelation therapy to combat the tendency to iron overload. Patients who inherit milder mutations may have β-thalassemia intermedia (TI). β-thalassemia heterozygotes (Thalassemia trait, TT) are normal or mildly anemic, with no clinical symptoms.[5]

The diversity of the β- thalassemias phenotypes likely relates to complex genetic interactions, many not yet defined that underline these conditions, together with monogenetic, environmental modulators or modifiers[6]. Primary modifiers include the broad spectrum of β -thalassemia alleles (more than 200 different alleles). Secondary modifiers include other genes involved in globin

chain synthesis e.g. α- thalassemia, hereditary persistent fetal Hb (HPFH) and other Hb variants known to modify the phenotype. Tertiary modifiers include loci not involved in globin synthesis but might modify the complications of the disease, these include genes affecting bilirubin level, genes involved in iron metabolism (Hereditary Hemochromatosis), genes contributing to bone affections and genes contributing to susceptibility to infection.[7]

Hereditary Hemochromatosis (HH) is an autosomal recessive disorder caused by mutations in the HFE gene. It is characterized by increased iron absorption and storage, resulting in progressive and multisystem oxidative organ damage[8]. Two common HFE gene missense mutations, C282Y and H63D, have been described to be associated with the disease with different contribution in different populations[9-12]. Several studies have tried to correlate iron status in thalassemic patients and the presence of one or both HFE gene mutations, C282Y and H63D[12-17]. To the best of our knowledge, there is no information regarding C282Y and H63D HFE gene mutation among Egyptian children with β-thalassemia. Only one publication[18] studied the prevalence of these two mutations in Egyptians with HCV liver cirrhosis. The objectives of the present study were to study the prevalence of these two HFE gene mutations in Egyptian β-thalassemic children and to evaluate the effect of these two mutations on their iron status.

PATIENTS AND METHODS

Subjects: Fifty nine β-thalassemic children regularly attending the pediatric hematology clinic in Menoufiya University Hospital either for transfusion and chelation (23 TM and 23 TI) or for follow up of Hb level and iron status (13 patients with TT) were randomly selected for this study.

Regarding TM patients they were 11 males and 12 females aged 3-21 years (mean 9±5.23 years). These patients were treated to maintain the pre-transfusional Hb level above 8 g/dl and post -transfusional Hb above 10 g/dl by regular red blood cell concentrates transfusion.

Thalassemia intermedia (TI) patients, were 23 patients who regularly attend our hematology clinic for follow up and blood transfusion when needed. They were 11 males and 12 females aged 4-19 years (mean 10.39±3.51 years). Twenty one of them were needed to be regularly transfused (every one or two months) to maintain their growth and only 2 were occasionally transfused.

Desferroxamine (DFO) 30-50mg/kg/day was administered by subcutaneous infusions over 8-10 hours 5 nights /week. DFO was usually started when serum ferritin approximated 1000 ng/ml[19]. Twenty one patients of TM group were under regular chelation and 2 did not need to start the chelation yet. Of TI patients, 18 patients were regularly chelated by DFO and 5 did not need to start the chelation yet. For each regularly transfused patient (both for thalassemia major or intermedia) the mean amount of transfused RBCs (ml/kg/year) was calculated for the last 3 years.

For the chelated patients they were selected as being well compliant with the chelation therapy. The mean index of observation (OI) for each patient during

the last three years was ≥ 80% which is calculated as follow: OI = (the actual received DFO dose ÷ the prescribed DFO dose) X100.[17]

Our study, also, involved 13 thalassemia trait children (4 males and 9 females) aged 3-18 years (mean, 8.50 ± 4 years). All of them were non anemic (mean Hb 11.89 ± 0.47 gm/dl) with no previous history of iron therapy. They were selected from the sibs of our thalassemia major and intermedia patients.

Fifty (28 males and 22 females) apparently healthy, Egyptian children aged 6.5 to 16.9 years (mean 10.24±2.96 years) were selected as a control group. Their Hb levels ranged from 12.0 to 14.5 g/dl (mean 13±0.7g/dl).Their serum ferritin levels were within normal range for their ages with mean of (82.2± 19.7 ng/ml) with normal Hb electrophoresis. The Ethics Committee approved the study and informed written consent was obtained from all patients and controls or their parents.

Evaluation of Iron Status: Serum ferritin level was used to estimate the iron status of our patients. The mean serum ferritin level in the previous 3 years was considered (on the average of 3 determinations yearly) for each patient. We excluded the results obtained when there was active liver disease (elevated ALT and AST) or any acute or chronic inflammatory illness. Serum ferritin was measured by Enzyme Linked Immune Sorbent Assay (ELISA) technique.

Detection of HFE Gene Mutations: Blood samples were drawn from patients with β- thalassemia major and intermedia immediately before their blood transfusion session.

DNA was extracted from both control's and patient's leukocytes according to the method of Al-Janabi and Martinez[20]. Two single Polymerase chain reactions (PCR) were performed to amplify relevant segments of the HFE gene that carry the H63D and C282Y mutation sites using the primers described by Feder et al[21]. PCR was performed using a Perkin Elmer Thermal Cycler Gen Amp 9700 (Applied Biosystems, UK). A total volume of 25 µl PCR reaction containing 100 ng of genomic DNA, 20 pmol of each primer, 200 µM of each dNTPs and 1.25 units of AmpliTaq Gold DNA polymerase (Applied Biosystems). PCR cycles were one cycle at 95°C for 10 minutes, to activate the AmpliTaq Gold and to initial denature the DNA, followed by 32 cycles of 94°C for 30 seconds, 58°C for 30 seconds and 72 °C for 30 seconds and a final extension step of 72°C for 5 minutes. Ten microleters of the PCR products were subsequently digested with the restriction enzymes RsaI (C282Y mutation) and MboI (H63D mutation). The digestion products were electrophoresed on a 3% gel (1:1 agarose/Nusiev). Amplification with the primers for codon 282 produced a 390 bp fragment which is cut into 250 and 140 bp fragments in the wild allele while the mutant allele will be cut into 250, 111 and 29 bp. On the other hand, the H63D mutation abolishes the MboI recognition site in the 208 bp PCR product: While normal DNA is cut into two fragments (70 and 138 bp), the mutated DNA is not cut.[22]

Statistics: Results were collected, tabulated, statistically analyzed by IBM personal computer and Statistical Package for the Social Sciences (SPSS) version 11. Differences between

and within the groups were evaluated by the non-parametric Mann-Whitney test. Chi-square test (χ^2) was used to study the relation between two qualitative variables. Kruskal- Wallis test (non -parametric test): Is a test of significance used for comparison between three or more groups not normally distributed having quantitative variables. Pearson - correlation (r): is a test used to measure the association between two quantitative variables. Multiple linear regression analysis test was used to study the risk factors -A P-value of < 0.05 was considered statistically significant.[23]

RESULTS:

The C282Y mutation was not present in any of the 59 β-thalassemia patients or the 50 controls. The H63D heterozygous state was detected in 15 (25.4%) thalassemic patients with allele frequency of 12.71% of the tested chromosomes and in 11 (22%) controls with allele frequency of 11%. There was no significant difference between the thalassemic group and the control group regarding the prevalence of H63D mutation. The prevalence of carriers for the H63D mutation was 26.1% with an allele frequency of 13.04% in patients with either β-thalassemia major or intermedia, while in β- thalassemia trait the prevalence of this mutation was 23.1% with an allele frequency of 11.54%. The age of first transfusion was significantly lower in TM in comparison with TI patients. On the other hand, the amount of transfused RBCs (ml/kg/year) was significantly higher in TM in comparison with TI patients. There were no significant differences between TM and TI patients regarding the mean serum ferritin level (Table 1).

Table 1: Clinical and laboratory data of the studied beta thalassemia group.

	Age (yr) Mean±SD	Age of 1st transfusion (yr) Mean±SD	Amount of transfused RBCs (ml/kg/yr) Mean±SD	Serum Ferritin (ng/ml) Mean±SD	OI (%) Mean±SD	Sex		Consanguinity		H63D genotype	
						Male	Female	+ ve	- ve	H/D	H/H
TM	9 ± 5.2	0.97 ± 0.4	191.8 ± 17.1	1599.6± 913.2 Median=1160	86.7 ± 4.6	11	12	6	17	6	17
TI	10.4 ± 3.5	5.5 ± 1.5	77.5 ± 13.9	1126.4± 559.8 Median=1000	89.2 ± 5.2	11	12	6	17	6	17
TT	8.5 ± 4.0	----------	----------	89.8±27.5 Median=98	-------	4	9	2	11	3	10
Control	10.24±2.96	----------	----------	82.2± 19.7	-------	28	22			11	39
P value	P>0.05	*P<0.001	*P<0.001	P1>0.05 *P2<0.001		P>0.05		P>0.05		P>0.05	

*= Significant, P1 = comparison between TM and TI, P2 = comparison between both TM and TI in one side and TT and the control in other side

H/D= Heterozygote for the mutant H63D allele , H/H =Homozygote for the normal wild H63D allele

In both TM and TI groups the mean serum ferritin (ng/ml) was significantly higher in patients who were H63D heterozygous compared to H63H homozygotes ($p < 0.05$). However there was no significant difference between H63D heterozygotes and H63H homozygotes regarding the age of first transfusion, the amount of transfused RBCs (ml/kg/year), the OI (%) or the presence of iron overload complications. On the other hand, there was no significant difference between mean serum ferritin in H63D heterozygotes and H63H homozygotes thalassemia trait patients (Table 2).

Table 2: Relationship between H63D genotype and serum ferritin, mean transfused RBCs volume, OI and the presence of complications in thalassemic patients.

	Thalassemia Major (TM)			Thalassemia intermedia (TI)			Thalassemia Trait (TT)	TM and TI groups
H63D genotype	Mean Serum Ferritin±SD (ng/ml)	Mean transfused RBCs±SD (ml/kg/yr)	OI (%) ± SD	Mean Serum Ferritin±SD (ng/ml)	Mean transfused RBCs±SD (ml/kg/yr)	Mean OI (%) ± SD	Mean Serum Ferritin±SD (ng/ml)	Presence of Complications [number (%)]
H/D	2288.2±820.5 Median=2485	187.5±7.6	87.5±4.2	1596.7±674 Median=1600	72.0±10.8	90.0±3.5	87.7±19.7	3 (6.5%)
H/H	1356.6±834.1 Median=1000	193.4±19.4	86.3±4.8	960.5±420.4 Median=1000	79.1±14.5	88.5±5.8	90.4±30.3	3 (6.5%)
P value	*P<0.01	P>0.05	P>0.05	*P<0.05	P>0.05	P>0.05	P>0.05	P>0.05

* = Significant

H/D= Heterozygote for the mutant H63D allele,

H/H =Homozygote for the normal wild H63D allele.

There were significantly positive correlations between serum ferritin levels and the age of patients in all thalassemia groups (TM, TI and TT) (Table 3).

Table 3: Correlation (r) between serum ferritin and age (yr), (for TM,TI&TT) age of 1st transfusion (yr), amount of transfused RBCs (ml/kg/yr) and OI (%) in thalassemia major and intermedia patients.

Correlation between ferritin level (ng/ml) and:	Thalassemia Major		Thalassemia Intermedia		Thalassemia Trait	
	r	P value	r	P value	r	P value
Age (yr)	0.83	* <0.001	0.62	* <0.05	0.75	*<0.01
Age of 1st transfusion	0.17	>0.05	0.57	>0.05		
Amount of transfused RBCs (ml/kg/yr)	0.25	>0.05	0.04	>0.05		
OI (%)	-0.35	>0.05	-0.04	>0.05		

* = Significant

Multiple linear regression analysis showed that the older age followed by the presence of H63D mutation in heterozygote state, (among both TM and TI) are the most important risk factors for iron overload, as indicated by serum ferritin level followed by the amount of transfused RBCs (ml/kg/yr) (Table 4).

Table 4: Multiple linear regression analysis for risk factors affecting serum ferritin in patients of both β-thalassemia major and intermedia.

	Un-standardized Coefficient		Standardized Coefficient	t	P
	B	SE	Beta		
Constant	-505.046	1398.893		-0.361	> 0.05
Age(yr)	132.034	16.628	0.731	7.94	*0.000
Amount of transfused BCs (ml/kg/yr)	5.915	1.222	0.444	4.839	*=0.000
OI (%)	-4.901	14.332	-0.031	-0.342	>0.05
H63D mutation	802.682	142.288	0.469	5.641	*=0.000

* = Significant

DISCUSSION

The allelic frequencies of C282Y and H63D are widely variable between different populations. That of C282Y ranged from 0% to 9.9% with this allele seen to be nearly 0% in North African population. On the other hand, the allelic frequency of H63D in different populations ranged from 0% to 20.4%[11,24-26]. In the present study, neither carrier for the C282Y allele nor homozygosity for H63D mutation was detected in any of studied controls or thalassemic patients. The prevalence of H63D mutation heterozygosity among controls was 22% (with allele frequency of 11%). For the studied thalassemia patients the prevalence of H63D heterozygosity was 25.42% with allele frequency of 12.71%. Published data regarding H63D and C282Y allele frequency among Egyptian population are scars. The only published data was that of Settin et al.[18] who studied HFE gene mutations in Egyptians with hepatitis C virus infection with liver cirrhosis. They stated that HFE gene analysis showed that the abnormal C282Y was not detected among patients or controls whereas; the prevalence of heterozygosity for H63D allele was noted to be similar in controls (21.2%) and in cases (20.0%). These results are in accordance with our results. In this study the prevalence of H63D heterozygosity among both TM and TI groups was 26.1% (6 out of 23 patients in each group) with H63D allele frequency of 13.04% (6 out of 46 tested chromosomes in each group). In individuals with β- thalassemia trait the prevalence of this mutation was 23.1% (3 out of 13 tested patients) with allele frequency of 11.54% (3 out of 26 tested chromosomes) with no significant difference in allele frequencies between the all studied groups (Table 1). There was no significant difference in C282Y or H63D allele frequencies between the thalassemic group as a whole or any of the thalassemia groups (TM,TI or TT) and the controls. This is in agreement with what was reported that there is no difference regarding HFE frequency among thalasemic patients (TM[4] or TT[15]) compared to normal population but against what was reported by others that HFE mutations C282Y and H63D are more frequent in BTM patients[14,17], in BTI[27] and in TT[12,14] than in the normal population.

Some of thalassemia patients develop iron overload even without erythrocyte transfusions. If transfusions are needed, they will add more to the body iron excess[28]. This discordant course between individuals with the same he-

matological alteration of thalassemia is very common and may be related to the different inherited mutations[12]. The presence of HFE gene mutations may adversely affect the course of a number of disorders including thalassemia. Data on the interaction of primary or hereditary hemochromatosis (HH) in thalassemic patients are scanty.[4]

Although it was shown to be a poor predictor of iron load as it is affected by other conditions like infections or inflammation, liver disease or vitamin C deficiency, periodic assessment of serum ferritin level is still the widely and most commonly used method to asses and monitor iron load in thalassemic children[29]. We considered it as the indicator of iron load in this study. Many similar studies consider the mean serum ferritin level as indicator of iron overload in thalassemic children tested for HFE mutations.[12,13,17]

The results revealed a statistically significant higher serum ferritin levels in patients with either β-thalassemia major or intermedia who were heterozygotes for H63D mutation compared to patients who did not have it (i.e. H63H homozygotes) (Table 2).

Data on the interaction of primary hemochromatosis gene (HFE gene) mutations in thalassemia patients are controversial. Some researchers showed that the coexistence of HFE mutations in thalassemic patients may lead to severe iron overload[12,15,27] while others did not[4,14,16,17,30,31]. This controversy may be due to differences in clinical status and inclusion criteria, especially the age since first transfusion, between different studies. Moreover, the presence of other unknown iron-related gene (s) or HFE mutations could play a role in populations with a low prevalence of the known HFE mutations.

Comparison of the severity of iron overload among transfused thalassemic patients (either major or intermedia) is a complex issue. Several factors influence the iron burden in these subjects, including the transfusion protocol and compliance of chelation therapy[4]. Using a homogenous transfusion protocol for every group and selection of good compliant patients for chelation (OI>80%) helped minimizing the effects of these variables. Moreover, the comparable Hb levels in patients with and without the mutation exclude the possibility that the difference in ferritin levels is due to the degree of anemia which may influence iron absorption. Our results confirm the hypothesis that in some way the H63D mutation could be implicated in increasing iron storage when in interaction with other genetic determinants.[32]

In this study there was no statistically significant difference in prevalence of iron overload complications in thalassemic patients (TM and TI) regarding H63D genotype (Table 2). It was recorded that although the levels of serum ferritin, serum iron and transferrin saturation are higher than normal in heterozygotes for HH, complications due to iron overload are extremely rare in these individuals[28]. This can be explained by the fact that these complications need long time to be manifested. Moreover, our studied patients were good chelated with good compliance for DFO, suggesting that the optimal medical treatment is able to overcome the potential adverse effect on iron absorption caused by the defective HFE gene.[33]

In this study, there was significant positive correlation between the age and the mean serum ferritin in each TM, TI and TT groups (Table 3). Moreover, the multiple linear regression analysis for risk factors affecting serum ferritin in our patients revealed that the most important risk factors for iron overload among transfused thalassemia patients (TM and TI) were older age followed by presence of the H63D mutation in the heterozygote state and then the amount of transfused RBCs (ml/kg/yr) ($t=7.94$ $p<0.000$, $t=5.641$ $p=0.000$, $t=4.839$ $p=0.000$ respectively). This reflects the cumulative effect of both transfusion (in transfused patients) and intestinal iron absorption as iron tends to increase with age.[34]

In our thalassemia trait patients there was no significant difference in the mean serum ferritin level between (H63D) and H63H patients (Table 2). This is in agreement with previous studies[14,16,31]. On the other hand, other studies showed that heterozygosity for H63D is a risk factor for iron overload in thalassemia trait patients[15]. While others stated that beta-thalassemia carriers who are homozygotes for the H63D mutation had higher ferritin levels than beta-thalassemia carriers with the H/H genotype, suggesting that the H63D mutation may have a modulating effect on iron absorption.[13]

CONCLUSION

There is no difference regarding the HFE mutations between thlassemic children compared to normal's and the presence of a heterozygous H63D mutation and older age are two risk factors for iron overload in Egyptian β-thalassemic children. Our study supports the emerging importance of genetic screening for this mutation in thalassemic children and in other iron-loading conditions. This may improve the quality of life in these patients by frequent follow up for earlier detection of iron overload and its complications and by implementing more aggressive iron chelating protocols.

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