Original Article

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Frequency of C282Y and H63D Mutations of HFE Gene and Their Correlation with Iron Status in Iranian Beta-Thalassemia Major Patients

Mohammad Soleiman Soltanpour PhD[1], Majid Farshdousti Hagh PhD[2], Koorosh Kamali PhD[3], Gholam Ali Jafari MSc[4], Kambiz Davari MD[5,*]

1. Department of Medical Laboratory Sciences, School of Paramedical Sciences, Zanjan University of Medical Sciences, Zanjan, Iran
2. Department of Hematology, Faculty of Medicine, Tabriz University of Medical Sciences, Tabriz, Iran
3. Department of Public Health, School of Public of Health, Zanjan University of Medical Sciences, Zanjan, Iran.
4. Department of Medical Laboratory Sciences, School of Paramedical Sciences, Zanjan University of Medical Sciences, Zanjan, Iran
5. Department of Pediatric, School of Medical Sciences, Zanjan University of Medical Sciences, Zanjan, Iran
*Corresponding author: Dr. Kambiz Davari, Mahdavi Boulevard, Department of Pediatric, School of Medical Sciences, Zanjan University of Medical sciences, Zanjan, Iran. E-mail: kdatzums@yahoo.com

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Abstract

Background: Co-inheritance of hemochromatosis (HFE) gene mutations may play an essential role in the pathogenesis of iron overload in beta-thalassemia major (BTM) patients. The present study aimed to investigate the prevalence of HFE C282Y and H63D mutations in BTM patients and their correlation with some demographic data and biochemical iron markers.

Materials and Methods: The study population consisted of 65 BTM patients and 200 apparently healthy matched controls. The genotyping of HFE gene mutations were conducted by Polymerase Chain Reaction-Restriction Fragment Length Polymorphism method. Plasma ferritin levels were determined by enzyme immunoassay. Plasma iron and transferrin levels were assessed by routine laboratory methods. Data analysis was performed using SPSS (version 22).

Results: The carrier frequency of the H63D mutation was 20% with an allele prevalence of 12.31% in BTM patients, while in the control subjects these values were 21% and 11.75%, respectively ($p>0.05$ for both). The HFE C282Y gene mutation was not detected in BTM patients and only detected in the 1.5% of controls. The carriers of HFE H63D mutation had significantly higher plasma ferritin levels, iron levels and transferrin saturation levels than non-carriers ($p=0.005$, $p=0.008$, $p=0.042$, respectively). Moreover, no significant differences were observed regarding the mean volume of transfused blood and splenectomy rate between BTM patients with and without HFE H63D mutation.

Conclusion: The present study demonstrated HFE H63D mutation as a significant contributing factor for iron overload in BTM patients. However, the genotype and allele distribution of HFE H63D and C282Y mutations didn't differ significantly between the two groups.

Key words: Beta-Thalassemia, C282Y, H63D, Hemochromatosis, HFE Mutation

Introduction

One of the most fatal diseases in human Beta-thalassemia major (BTM) is an autosomal recessive disorder characterized by decreased synthesis of hemoglobin and severe anemia. Regular red blood cell transfusion therapy is the main treatment for these patients that causes several adverse complications such as alloimmunization to erythrocyte antigens and iron overloading (1,2). So, secondary hemochromatosis due to regular red blood cell transfusion and also increased intestinal iron absorption is a common finding in BTM patients (1). Excess iron accumulation had toxic effects on many cells of the body that causes multiple organ damages such as diabetes, heart disease and cirrhosis (3). Iron overload is a common finding in thalassemia major patients. However, the severity of iron overload is variable in BTM patients so

that in a proportion of these patients the clinical and laboratory presentation of iron overload is more severe than that of other patients (4). So, the phenotypic variability of iron overloads observed in BTM patients may results from some genetic variations altering the iron metabolism in these patients. Among the probable causes, HFE gene mutations (C282Y and H63D) seem to be the most probable cause (5). The HFE gene located on chromosome 6 and encodes an HLA class I-like protein that complexes with beta 2- microglobulin and transferrin receptor (TfR) on the cell surface (6). HFE protein decreases the affinity of TfR for transferrin and lowers iron absorption by the cells (6).Two missense mutations (C282Y, H63D) have been identified in the HFE gene that cause defective interaction between HFE molecule, b2-microglobulin and TfR on the cell that result in iron accumulation and secondary hemochromatosis (7).

The C282Y mutation is the result of a G-to-A transition at nucleotide 845 which changes cysteine to tyrosine at the amino acid position 282 of the HFE protein (7). The HFE H63D mutation is due to a C-to-G change at position 187 that converts histidine to aspartic acid at codon 63 of HFE the protein (7). The aim of this study was to investigate the genotypic and allelic frequencies of HFE gene mutations C282Y and H63D in beta-thalassemia major patients in comparison to a normal control group. Moreover, the phenotypic expression of HFE genotypes on some hematologic parameters and biochemical iron markers was evaluated.

Materials and Methods

During the 2016 year, sixty five BTM patients including 34 males and 31 females who were on regular red blood cell transfusion were included in this study. The patients were selected from Zanjan and Tabriz cities of Iran. Also, 200 healthy control subjects including 108 males and 92 females were included in the study. The control subjects were included in the study if they had a similar age, sex and ethnicity with the patients group. All patients who were β-thalassemia major requiring repeated blood transfusions (at least 2 per month) were included in the study. Patients suffering from hepatitis B or hepatitis C infection or any other disease such as malignancy, renal disease, inflammation and autoimmune disorders were excluded from the study. Also, patients with thalassemia minor and intermedia or patients who refuse to be part of the study were not included in the study. Out of the 65 BTM, 17 (26.15%) patients were splenectomized and 11 patients (16.92%) had developed alloantibody with anti-K as the most common one. All of the 65 BTM were transfused with leukoreduced packed red blood cells. The mean hemoglobin levels of BTM patients were 8.1± 1.2 g/dl. The majority of the BTM patients were under regular chelation therapy with Desferroxamine (30-50mg/kg/day) that was administered by subcutaneously. Five of the BTM patients that had intolerance to Desferroxamine, were chelated with oral iron chelator Deferosirox. For molecular and biochemical analysis, fasting blood samples were collected from all subjects. DNA was extracted from blood leukocytes using a commercially available DNA extraction kit (Geno Plus Genomic DNA Mini, Viogene, Poland) according to the manufacturer's instructions. Detection of HFE C282Y and H63D mutations were performed by Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) method using RsaI and BclI restriction enzymes, respectively, as described previously (8). The size of PCR product for HFE C282Y mutation was 390bp and its digestion with RsaI enzyme results in 250bp and 140bp fragments in the presence of wild allele and 250bp, 111bp and 29bp fragments in the presence of mutant allele. Also, the size of amplified PCR product for HFE H63D was 207bp that following digestion with BclI enzyme produces 138bp and

69bp in the presence of wild allele and a single non-cleaved 207bp band in the presence of mutant allele (Figures 1A and 1B). Serum Iron and transferrin levels were measured by routine colorimetric methods by commercially available kits (Pars. Azmoon Ltd., Tehran, Iran) using Mindray biochemical auto-analyzer (High-tech Industrial Park, Nanshan, Shenzhen 518057, P.R. China.). Transferrin saturation index was calculated by the ratio of serum iron and total iron-binding capacity, multiplied by 100. Ferritin levels were determined by ELISA kit (Pishtaz Teb Ltd, Tehran, Iran) according to the manufacturer's protocol. Informed consent was obtained from all patients and controls included in the study. The study was approved by the ethical committee of Zanjan University of Medical Science (Ethical committee code: ZUMS.REC.1393.166), Zanjan, Iran. Statistical analysis was done by SPSS (version 22). Categorical variables were analyzed by Chi-square test or Fisher exacts test and continuous variables were examined using student t-test.

Results

In current study, the C282Y mutation was not detected in any of the 65 BTM patients and only was detected in 1.5% of the control subjects in a heterozygote manner. The H63D mutation was detected in 13 BTM patients (20%) among whom three individuals (4.62%) were homozygote for H63D mutation. Among 200 control subjects, 42 individuals had *HFE* H63D mutation (21%), of whom 5 individuals (2.50%) were homozygote for H63D mutation. The mutant allele frequency of *HFE* H63D mutation was 12.31% and 11.75% in BTM patients and control subjects, respectively (OR=1.05; %95 CI: 0.57-1.93, p=0.864). Statistical analysis using *Chi*-square test showed no significant differences in genotypic distribution of *HFE* H63D mutation between BTM patients and controls (p>0.05; Table I). Moreover, analysis of *HFE* H63D mutation under dominant (HD+DD vs. HH) (OR=0.94; %95CI: 0.46-1.88, p=0.862) or recessive (DD vs. HH + HD) (OR=1.88; %95CI: 0.44-8.12, p=0.394) genetic models indicated no significant differences between BTM patient and control subjects regarding the distribution of different genotypes, as shown in Table II. The correlation of *HFE* H63D mutation with some biochemical parameters indicated that plasma ferritin levels (p=0.005), iron levels (p=0.008) and transferrin saturation levels (p=0.042) were significantly higher in carriers of the *HFE* H63D mutation than non-carriers. However, no significant differences were observed in the sex distribution, average volume of annually transfused blood and splenectomy rate between BTM patients with and without *HFE* H63D mutation (p>0.05) (Table III). Also, comparing the age distribution between patients with and without *HFE* H63D mutation revealed no significant differences between the two groups (p>0.05). Moreover, no significant differences were observed between patients with and without *HFE* H63D mutation regarding the type, duration and dosage of chelation drugs (p>0.05). Table IV represents the genotype frequency of *HFE* H63D and C282Y gene mutations among thalassemia patients in different ethnic populations.

Table I: Genotype frequency of HFE H63D and C282Y gene mutations among thalassemia major patients and controls

Mutation	Patients (n=65)	Controls (n=200)	OR (95%CI)	*p* value
***HFE* H63D**				
Wild	52 (80.00%)	158 (79.00%)	1	Ref
Heterozygote	10 (15 .38%)	37 (18.50%)	0.82 (0.38-1.77)	0.614
Homozygote	03 (04.62%)	05 (2.50%)	1.82(0.42-7.89)	0.421
***HFE* C282Y**				
Wild	65 (100%)	197 (98.5%)	1	Ref
Heterozygote	0 (0.0%)	03 (1.50%)	undefined	0.857*
Homozygote	0 (0.0%)	0 (0.00%)	undefined	undefined

*Fisher exact test

Table II: Analysis of HFE H63D gene mutation among thalassemia major patients and controls using dominant, recessive and allelic genetic models

Genetic model	Genotype/Allele	Patients (n=65)	Controls (n=200)	OR (95%CI)	*p* value
Dominant model	HH	52 (80.00%)	158 (79.0%)	1	Ref
HD + DD vs. HH	HD + DD	13 (20.0%)	42 (21.0%)	0.94 (0.46-1.88)	0.862
Recessive model	HD + HH	62 (95.38%)	195 (97.5%)	1	Ref
DD vs. HD + HH	DD	03 (4.62%)	5 (2.5%)	1.88 (0.44-8.12)	0.394
Allele model	H	114(87.69%)	353(88.25%)	1	Ref
H vs. D	D	16 (12.31%)	47 (11.75%)	1.05 (0.57-1.93)	0.864

H: wild allele, D: mutant allele

Table III: Profile of β-thalassemia major patients with and without HFE H63D mutation

Parameters	*HFE* H63D carrier (n= 13)	*HFE* H63D non-carrier (n=52)	*p* value
Ferritin (µg/L)	1930 ± 912	1221 ± 751	0.005
Iron (µg/dL)	213.68±48.46	165.87±57.80	0.008
TSI (%)	63.34±15.37	46.54± 14.23	0.042
Sex (M/F)	7/6	27/25	0.901
MTB (ml/kg/year)	225.82± 53.47	203.52± 63.34	0.247
Splenectomy	5/13	12/52	0.260

TSI: Transferrin saturation index, MTB: Mean transfused blood

Table IV: Genotype frequency of HFE H63D and C282Y gene mutations among thalassemia patients or carriers in different ethnic populations

Population (Reference)	No of cases	H63D (%)		C282Y (%)	
		Heterozygote	Homozygote	Heterozygote	Homozygote
Brazil (9)	168	22.61	2.38	4.76	0
Turkey (10)	33	15.15	3.03	0	0
North Indian (11)	308	13.96	0.97	0	0
Italy (12)	71	21	1.4	1.4	0
Tunisian (13)	50	26	04	0	0
Portugal (14)	101	30.7	0	2.97	0
Egypt (4)	75	37.3	10.7	0	0
Iran (current study)	**65**	**15.38**	**4.62**	**0**	**0**

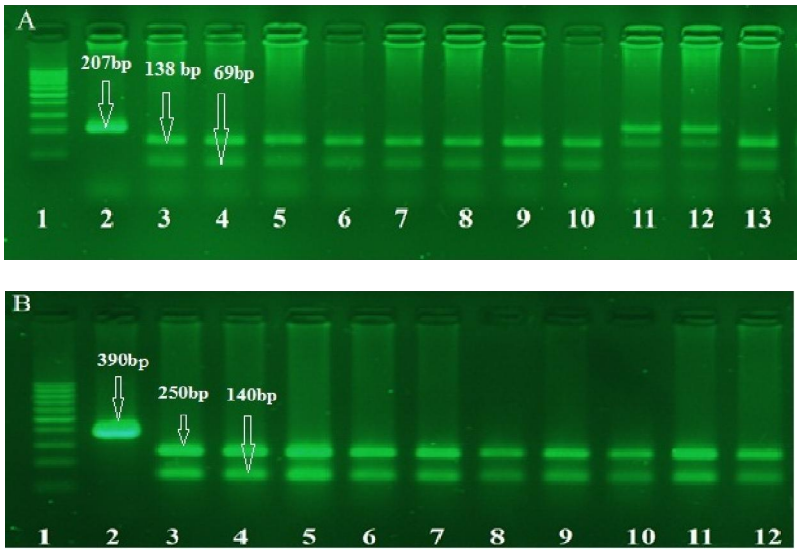

Figure1. (A) Mutation analysis of *HFE* H63D: The PCR products were digested by restriction enzyme BclI I: lane 1 (100 bp ladder); Lanes 3-10,13 wild type genotype (138bp, 69bp); Lanes 11, 12 heterozygote genotype (207 bp ,138 bp,69 bp); Lane 2 homozygote genotype (207bp). (B) Mutation analysis of *HFE* C282Y: The PCR products were digested by restriction enzyme RsaI I: lane 1 (100 bp ladder); Lane 2: Undigested product (390bp); Lanes 2-12 wild type genotype (250 bp, 140 bp).

Discussion

Identification of genetic mutations aggravating iron overload in BTM patients may be useful in proper management of iron overload and secondary hemochromatosis in these patients. Co-inheritance of HFE gene mutations may play an important role in the pathogenesis of iron overload in BTM patients. We hereby report the prevalence of HFE H63D and C282Y mutations among a group of 65 Iranian BTM patients and 200 healthy controls. The carrier frequency found for H63D and C282Y mutations were 20% and 0% among BTM patients and 21% and 1.5% among control subjects, respectively. So, no significant differences were observed in the carrier frequencies of HFE H63D and C282Y mutations between BTM patients and controls. The similar result was obtained by Turedi et al. (2013) in a study of 33 Turkish patients with beta thalassemia major who reported a carrier frequency of 18.18% and 0% for H63D and C282Y mutations, respectively (10). Also, the allelic frequencies found for H63D (12.31%) and C282Y (0%) mutations in present study were similar to the values obtained by Mokhtar et al, who reported an allelic frequency of 12.5% for HFE H63D mutation among Egyptian beta thalassemia patients (15). Also, as indicated in Table IV, the prevalence of heterozygote and homozygote genotypes of HFE H63D (15.38%, 4.62%) and C282Y (0%, 0%) mutations were similar with the reported frequency from Turkish population (10).

The results of present study are in agreement with the some previously published studies that found no significant differences in genotypic and allelic distribution of HFE H63D and C282Y mutations between BTM patients and controls (15, 16). However, some studies reported increased frequency of HFE H63D and C282Y mutations in thalassemia patients or carriers. For example, in a study by Mokhtar et al, it was found that HFE H63D mutation was more prevalent in beta-thalassemia carriers than control subjects, however, the significance level was very small (24% vs. 12.5%; p=0.049). Moreover, Oliveria et al found a significant difference in the frequency of C282Y mutation but not of the H63D mutation between beta-thalassemia carrier and control groups (9). In another study carried out by Sharma and colleagues a significant difference was observed in the frequency of heterozygous genotype of HFE H63D mutation between thalassemia patients and healthy controls (25.8% vs. 7.5%, $p<0.05$) (17). Numerous factors such as sample size and ethnic and racial differences may explain the conflicting results of association studies (18).

One of the main purposes of the present study was to evaluate the effect of HFE H63D mutation on the iron biochemical markers in BTM patients. Results indicated that the presence of HFE H63D mutation aggravated the individuals' iron status manifested by higher plasma ferritin levels, plasma iron and transferrin saturation levels compared with the wild-type group. So, according to the present study, the co-inheritance of HFE H63D mutation by some BTM patients may explain to some extent the phenotypic variability of iron overload and insufficient response to iron chelation therapy observed in some of these patients.

Similarly, in a study by El-Rashidi et al, it was shown that mean serum ferritin levels were significantly higher in both β-thalassemia major and β-thalassemia minor patients who are heterozygotes for HFE H63D mutation compared to those without this mutation (19). Also, Melis et al have reported statistically significant higher levels of serum ferritin in carrier of HFE H63D mutation than non-carriers in beta thalassemia individuals (20).

However, some other studies didn't confirm such an association (16, 21). The possible reasons for these contradictory results may be related to the variation in

study design such as sample size, sample selection criteria and also the presence of gene-gene and gene-environment interactions in the various studied populations (18).

The average volume of blood transfused per year, age, sex, type and dosage of chelation therapy and also splenectomy may have some effects on serum iron and ferritin levels (22). So, we investigated these parameters in BTM patients with and without HFE H63D mutation. Results indicated no significant differences between BTM patients with and without HFE H63D mutation regarding the amount of annually transfused blood, age, sex distribution, type and dosage of chelation drugs and splenectomy rate. Similarly, a study by Sharma et al indicated that in spite of higher prevalence of hyperferritinemia (as defined by serum ferritin levels greater than 500 ng/dl) in BTM patients with HFE H63D mutation compared with BTM patients without HFE H63D mutation (100% vs. 28.6 %, p = 0.002), no significant differences was seen regarding the age and the number of transfusions taken between the two groups of patients (17). Moreover, in agreement to our results, Martinez et al reported no significant differences between BTM patients with or without HFE mutations regarding the number of transfusions, splenectomy and chelation therapy (23).

Periodic assessment of serum ferritin levels was shown to be the most commonly used and easy access assay for evaluation of iron load in thalassemic children (24). Generally, there is a good correlation between serum ferritin levels and the extent of body iron stores; however, some confounding factors may influence the correlation between ferritin levels and body iron stores (24). A false increase in serum ferritin levels without any excess iron body may be seen in some acute or chronic inflammatory responses including neoplasia, chronic renal insufficiency, liver disease, autoimmune diseases and metabolic syndrome (24,25). Transferrin saturation levels may differentiate actual and false hyperferritinemia in these conditions. Unlike pseudo hypeferritinemia, in actual iron overload conditions the increase in fer¬ritin concentration is associated with increased transferrin saturation levels, similar to those observed in current study (26). So in the absence of acute or chronic inflammatory conditions serum ferritin levels may be used as a useful marker for assessment of body iron stores.

Conclusion

Iron overload is a common finding in BTM patients. Iron overload was more common and severe in carriers of HFE H63D mutation than non-carriers that may indicate the necessity for more intensive iron chelation therapy in such patients. However, no significant differences were observed between BTM patients and control subjects regarding the frequency of HFE H63D and C282Y mutations.

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Conflict of interest

There is no conflict of interest to be declared.

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