Effect of the Hemochromatosis Mutations on Iron Overload among the Indian β Thalassemia Carriers

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Background: Hereditary hemochromatosis is a disorder of iron metabolism characterized by increased iron absorption. HFE gene mutations C282Y and H63D are responsible for the majority of hereditary hemochromatosis cases. **Methods:** We tried to look at the effect of HFE mutations on the iron status. A total of 100 β thalassemia traits (BTT) with 100 normal individuals were screened for the C282Y and H63D mutations using PCR-RFLP. The serum ferritin levels were determined using ELISA kit. **Results:** We did not find the C282Y mutation in our study group. The allelic frequencies for H63D mutation did not differ significantly between β -thalassemia traits (8.5%) and normal controls (9%). BTT with H63D genotype of H/D

(143.16 $\pm$ 80.3 ng/ml) and D/D (504 ng/ml) showed higher ferritin levels as against H/H genotype (88.64 $\pm$ 92.43 ng/ml). The statistically significant difference was observed in the mean serum ferritin levels among the individuals showing H/H and D/D genotypes ($P < 0.002$) and H/D and D/D genotype ($P < 0.01$) in both the groups. **Conclusion:** This suggests that iron load in BTT tends to be aggravated with the co-inheritance of the H63D mutation. The mutant H63D gene showed the presence of haplotype 6 which is reported in the European population suggesting a common origin. J. Clin. Lab. Anal. 31: e22054, 2017. © 2016 Wiley Periodicals, Inc.

Key words: HFE gene mutations; India; iron overload; β thalassemia

INTRODUCTION

Hereditary hemochromatosis is a common autosomal recessive disorder of iron metabolism that results in progressive iron overload characterized by inappropriately high intestinal iron absorption. Two mutations have been identified in HFE hemochromatosis gene. A missense mutation causes a cysteine to tyrosine substitution at position 282 of the protein (C282Y). A second mutation changes a histidine residue at codon 63 to aspartic acid (H63D). More than 80% of HH patients in populations of European origin are homozygotes for a single mutation C282Y, or compound heterozygous for the C282Y and H63D mutations in the HFE gene. However, in the majority of Asian, Indian subcontinent, African, Australasian, and Amerindian populations, frequencies of the C282Y mutation are close to zero (1).

β -thalassemia trait is characterized by mild, ineffective erythropoiesis that can induce excess iron absorption and ultimately lead to iron overload (2).

However, only a minority of individuals develop iron overload indicating that other factors are involved in this phenotypic variability (3). Some authors have suggested that iron overload is the result of synergistic effect of the β -thalassemia trait and presence of hemochromatosis (HH) mutations, (4, 5) but the data are controversial (6).

In India, limited data are available on the prevalence of these genes. The primary aim of this study was to find out the allelic frequencies of these mutations in the Indian population and to evaluate the effect of HFE gene mutations C282Y and H63D on the iron status of β -thalassemia carriers.

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MATERIALS AND METHODS

The study was undertaken in 100 Indian β -thalassemia heterozygotes (mean age 39.4 SD $\pm$ 12.3) who came to our Institute for screening of hemoglobinopathies or were referred to us for prenatal diagnosis of thalassemia. One hundred healthy normal individuals (mean age 44.5 SD $\pm$ 15.6) were selected as control group. Written informed consent was obtained from all subjects. The study was approved by independent review board [Institutional ethical committee].

Red cell indices were measured on an automated blood cell counter Sysmex K 1000 (Sysmex Corporation, Kobe, Japan). HbA₂ levels were measured using cation exchange HPLC on the Variant Hemoglobin Testing System (Bio-Rad Laboratories, Inc., Hercules, CA). Genomic DNA was isolated from peripheral blood leucocytes using the QIA amp Blood Mini Kit. β Thalassemia mutations were characterized by Covalent reverse dot blot hybridization (CRDB) or amplification refractory mutation system (ARMS; 7). The detection of the C282Y and H63D mutations in HFE gene were carried out by PCR and restriction enzyme digestion using RsaI and MboI, respectively, as described by Lin et al. (8). The HFE haplotypes were studied using five restriction sites: -984 (G $\rightarrow$ C), IVS 2 (+5) C $\rightarrow$ T, IVS 4(-44) T $\rightarrow$ C, IVS5(-47) G $\rightarrow$ A, and Poly A +5 (C $\rightarrow$ T) described by Rochette et al. (9).

Statistical Analysis

Continuous variables were expressed as mean $\pm$ SD. The χ^2 test was used for the categorical variables as needed. Statistical significance was set at $P < 0.05$.

RESULT

Diagnosis of the β -thalassemia trait was defined below mean corpuscular volume (MCV < 80 fl), low mean corpuscular hemoglobin (MCH < 27 pg), and increased HbA₂ ($> 4.0\%$). Control individuals were characterized by normal hematological parameters and HbA₂ ($< 3.5\%$).

β Thalassemia Genotypes

All β -thalassemia carriers were heterozygous for one of the most common β thalassemia mutations described in the Indian population: 71% were carriers of the IVS nt 5 G $\rightarrow$ C mutation, 11% had CD30 G $\rightarrow$ C, 8% had CD15 (G $\rightarrow$ A), 4% were carriers of the IVS 1 nt 1 (G $\rightarrow$ T), and 3% each had CD 41/42 (-CTTT) and CD 8/9(+G) mutations.

HFE Allelic Frequencies and Genotypes

The C282Y mutation was not detected in both the groups (β thalassemia carriers and normal) studied. Of the 100 normal individuals studied, 14 were heterozygotes for the H63D mutation (H/D) and 2 were D/D homozygotes giving an allele frequency of 9% (Table 1). Sixteen of the 100 β -thalassemia carriers were heterozygotes for the H63D mutation (H/D) and 1 was D/D homozygous. The allelic frequency of the H63D mutation in the β thalassemia carrier group was 8.5%. Overall incidence of H63D mutation among the population was 8.75%.

Mean serum ferritin and hemoglobin levels were calculated in each group. Table 1 shows these values according to the H63D genotype in the control and the β thalassemia carrier group. Among both the study groups, a statistically significant difference was detected between the mean serum ferritin values in the H/H and D/D groups ($P < 0.002$) and between the H/D and D/D ($P < 0.01$). No statistically significant difference was detected in serum ferritin values between the H/H and H/D groups. The mean hemoglobin concentration according to the H63D genotype among both the groups did not show statistically significant differences.

Haplotype analysis of all the H63D mutant allele showed presence of the GCTAC [haplotype 6] sequence for five restriction sites tested. All three homozygotes of the H63D mutation showed homozygosity for haplotype 6 which is identical to that seen in Europeans.

DISCUSSION

Hereditary hemochromatosis is considered to be the most commonly inherited disorder in Caucasians and presents variable prevalence among different ethnic groups (10). The global prevalence of the C282Y missense point mutation varies considerably among the populations studied (11). It is most prevalent in western European populations. The allele frequency in most European countries varies between 10% and 20%, with higher frequencies being reported in northern countries around the Mediterranean and decreasing with the distance from the Mediterranean region. In contrast, the C282Y mutation is absent in Asian, African, and Australian indigenous populations (12, 13). The present study confirms the absence of the C282Y mutation among Indian population. Similar observation was reported by Thakur et al. (14) and Agarwal et al. (15). The only study from India which reported the presence of the C282Y mutation was from North India (16). In this study the C282Y

TABLE 1. Serum Ferritin and Hemoglobin Values in β -Thalassemia Heterozygotes According to Their H63D Genotype

Genotype H63D	β thalassemia carriers ($n = 100$)			Normal ($n = 100$)		
	No of subjects (%)	* Ferritin levels $\mu\text{g/ml}$ Mean $\pm$ SD	Hb g/dl Mean $\pm$ SD	No of subjects (%)	§ Ferritin levels ng/ml Mean $\pm$ SD	Hb g/dl Mean $\pm$ SD
H/H	83 (83)	88.64 $\pm$ 92.43	11.20 $\pm$ 2.51	84 (84)	75.80 $\pm$ 58.9	13.0 $\pm$ 2.12
H/D	16 (16)	143.16 $\pm$ 80.3	10.07 $\pm$ 2.81	14 (14)	103 $\pm$ 81.29	13.81 $\pm$ 1.03
D/D	1 (1)	504	14.4	2 (2)	517.5 $\pm$ 116.6	14.05 $\pm$ 0.63

NS, Non-significant.

* P values: H/H vs. H/D–NS; H/D vs. D/D – $P = 0.01$; H/H vs. D/D – $P = 0.002$.§ P values: H/H vs. H/D–NS; H/D vs. D/D – $P = 0.005$; H/H vs. D/D – $P = 0.001$.

mutation was reported in 1 of 59 healthy individuals (1.7%).

The H63D mutation has been found in diverse ethnic back grounds and may be an old mutation (11). A study carried out by Leão GD in patients with persistent increase of serum ferritin from northeastern Brazil showed 31.44% patients were heterozygous for H63D mutation while 8.03% patients were homozygous for H63D mutation. They concluded that due to the high prevalence of hemochromatosis, its genetic diagnosis has become a challenge, especially in the high-risk group (17). H63D is the major mutation present in the Indian population ranging from 9.1% to 13.9% (15, 18). Our results also showed quite a high prevalence of the H63D mutation (8.75%) among the Indian population. Its clinical significance is not that clear as that of the C282Y mutation. There are conflicting data as to whether it contributes to iron overload in heterozygotes. The three H63D homozygous individuals in our series had iron overload, but to a degree that was not severe enough to cause symptoms. Two independent pathways have been proposed for iron metabolism, the erythroid regulator, which modulates intestinal iron absorption, and the storage regulator, which controls iron accumulation. There are reports which showed that the erythroid regulator (β -thalassemia) is more pronounced than the storage regulator (the mutated HFE gene) in determining the degree of iron absorption (19, 20). This hypothesis shows that β -thalassemia carriers might exhibit an advantage in balancing iron storage in their organs. Nevertheless, published data indicate more severe hemochromatosis symptoms when heterozygosity for the C282Y mutation is associated with β -thalassemia (21) and when higher serum iron levels in co-inherited β -thalassemia are associated with heterozygosity and homozygosity for H63D (4). Ruiz-Argüelles et al. (2001) have described a propositus, who on co-inheriting beta-thalassemia and H63D mutation of HFE gene (both in heterozygous condition) developed severe iron overload with chronic liver failure and portal hypertension (22). However, these

results remain controversial (23). Though no statistically significant difference was detected in serum ferritin values between the H/H and H/D groups in our thalassemia heterozygote group, we did observe that mean serum ferritin levels were increased in β thalassemia carriers heterozygous (H/D, 143.16 $\pm$ 80.3 $\mu\text{g/dl}$) and homozygous (D/D, 504 $\mu\text{g/dl}$) for the H63D mutation as compared to individuals showing absence of this mutation (H/H, 88.64 $\pm$ 92.43 $\mu\text{g/dl}$). These results suggest that iron load in β -thalassemia trait tends to be aggravated with the coinheritance of the H63D mutation, even when present in the heterozygous state. The study of Martins et al. (5) also showed an increase in serum iron ($P = 0.029$) and transferrin saturation ($P = 0.009$) in β thalassemia carriers heterozygous for the H63D mutation. Melis et al. (4) found significantly higher levels of ferritin in β -thalassemia carriers who were homozygous for the H63D mutation but found no statistical difference in heterozygous H63D individuals. They suggested that the H63D mutation may have a modulating effect on iron absorption. Similar results were obtained in our study. The other studies in Indian population by Kaur et al. (16) and Garewal et al. (24) indicated that the H63D mutation did not influence the degree of iron load. Some studies in the Mediterranean population were of the same opinion that β thalassemia carriers with heterozygosity for the H63D or C282Y mutations were not associated with iron overload. (6, 23). A study by Kelley et al. (25) on patients homozygote for H63D showed that though the mutation is associated with an elevated mean ferritin level, only 6.7% of the patients documented iron overload at follow-up. An over view on genetics and clinical implications of HFE-associated hereditary hemochromatosis by Emanuele et al. (26) reported that knowledge of HFE-HH is essential so that one can identify individuals at risk and provide appropriate management of care and referral.

The study of haplotype helps us to determine the pattern of population migration and admixture. Our results of the haplotype of the mutant allele of

H63D revealed only presence of Haplotype 6 (GCTAC) which is identical to that seen in Europeans. Similar observations were reported among the North Indian population (16, 24). Therefore, it seems that in the Indian population, this mutation might have occurred once due to population admixture with Europeans.

Rochette et al. (9) showed linkage of the H63D mutation with three different haplotypes, i.e. haplotype 1 (TTG), haplotype 2 (TTA) and haplotype 5(CTG) in Sri Lankan population. These findings suggest that the H63D mutation in Sri Lanka has a different origin than that of India.

The population genetic screening to prevent HFE-associated hereditary hemochromatosis can be practicable and acceptable. Although the reports of this disease are mere, more studies are required to investigate the prevalence of this disease and association with other diseases among the Indian population.

CONCLUSION

Our overall results demonstrate that the β -thalassemia carriers tend to be aggravated with the coinheritance of the H63D mutation, even when present in heterozygous state. Haplotyping of the mutant H63D allele among all the Indian studies confirms its linkage with the European haplotype 6 (CTA) and a single origin of this mutation in India.

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AUTHOR'S CONTRIBUTION

All authors have contributed sufficiently to the project to be included as authors. AS, SC, and PH performed the research work; AN designed the research study and wrote the article; RC and KG contributed in data analysis.

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