AIMS Molecular Science, 8(4): 233–247.
DOI: 10.3934/molsci.2021018
Received: 09 September 2021
Accepted: 09 November 2021
Published: 12 November 2021

http://www.aimspress.com/journal/Molecular

Research article

Frequency of hereditary hemochromatosis gene mutations and their effects on iron overload among beta thalassemia patients of Chennai residents

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Abstract: Hereditary Hemochromatosis (HH) is an autosomal recessive disorder of iron metabolism associated with *HFE* gene mutations, characterized by increased iron absorption and accumulation leading to multi-organ damage caused by iron overload toxicity. Beta thalassemia is caused by a mutation in the human beta globin gene. Imbalanced production of globin chain results in beta thalassemia, where the unpaired alpha chains precipitates in red cell precursors leading to ineffective erythropoiesis and reduced RBC survival. Both HH and beta thalassemia condition results in rapid accumulation of iron lead to iron overload in tissues and organs. The study aims to analyze the frequency of *HFE* variants among beta thalassemia cases and their effect on iron overload. The frequency of three *HFE* variants C282Y, H63D, S65C was analyzed by PCR RFLP method among Beta Thalassemia Trait (BTT) (n = 203), Beta Thalassemia Major (BTM) (n = 19) and age and sex-matched control samples (n = 200). The present study furnished allele frequency of H63D variant in BTT, BTM and controls 8.13, 15.8 and 6% respectively. Ten out of 33 heterozygous H63D variants exhibited iron overload with higher ferritin levels indicating *HFE* variant might aggravate the absorption of iron. The C282Y variant was present in heterozygous state in 1 case among beta thalassemia carriers. The C282Y variant was absent among BTM and control cases. S65C *HFE* variant was absent in the present study.

Iron overload was completely absent in the control cases among all three *HFE* genotypes. Hence it is inferred from the present investigation, analysis of *HFE* genes and iron status will remarkably help to reason out the probable reason behind the iron status and support in proper management of beta thalassemia cases.

Keywords: Hereditary Hemochromatosis; beta thalassemia; *HFE*; iron overload; ferritin

Abbrevations: BTH: Beta Thalassemia Homozygous; BTM: Beta Thalassemia Major; BTT: Beta Thalassemia Trait; *HBB*: Hemoglobin Subunit Beta gene; HH: Hereditary Hemochromatosis; SNPs: Single Nucleotide Polymorphism; WT: Wild type allele

1. Introduction

HFE gene the causative factor for HH is located on the short arm of chromosome 6. There are four types of HH but type 1 hemochromatosis is the commonest disorder. The *HFE* gene regulates the production of hepcidin protein which is the master iron regulatory hormone. HH is caused by missense mutations—single nucleotide polymorphism (SNPs) in the *HFE* gene which includes the common mutations. When the *HFE* gene is affected with C282Y, H63D, S65C variants, the protein causes reduced synthesis of hepcidin expression in response to iron, thereby uncontrolled iron regulation and increase in plasma iron concentration occurs [1]. First C282Y variant [rs1800562; NM_000410.3 (*HFE*): c.845G>A (p.Cys282Tyr)] results from a single base substitution of cysteine to tyrosine at 282 position. The second H63D variant [rs1799945; NM_139011.3 (*HFE*): c.77-2168C>G (p.His63Asp)] involves the 63rd position where histidine is replaced by aspartate. The third gene variant is caused by the substitution of serine to cysteine at position 65–S65C [rs1800730; NM_000410.3 (*HFE*): c.193A>T (p.Ser65Cys)] [2–4].

The C282Y variant is highly prevalent in European countries and North America. Around 9.0% frequency of C282Y heterozygous is noted. Less than 1.0% population carries homozygous C282Y variant. Very low frequency of heterozygous C282Y variant around 0–0.5% and nil homozygous C282Y variant were reported in Asians, Indian subcontinent, African, Middle East, and Australians [6]. The heterozygosity and homozygosity for H63D were found to be 19.4% and 1.9% globally. The heterozygosity for H63D in the Indian subcontinent was 15.1% [6]. The S65C variant causes only milder forms of HH disease.

Beta thalassemia condition involves mild, ineffective erythropoiesis leading to anemia which in turn causes excess iron absorption and iron overload [7]. More than 400 causative mutations have been characterized in the hemoglobin subunit beta (*HBB*) gene. These causative mutations are present in the beta globin gene or the regulatory region [8]. Thereby in beta thalassemia cases even without blood transfusion iron overload develop in patients [9].

HH is a rare condition in India; however thorough investigation of HH among beta thalassemia carriers and beta thalassemia major condition is not explored completely in India [10]. In India, few studies and limited data pertaining to the effect of hereditary hemochromatosis among beta thalassemia

have been done. Thereby, it is proposed to investigate the frequency of *HFE* variants among beta thalassemia cases and their effect on iron overload.

2. Material and methods

The study included patients referred by clinicians for diagnosis/screening of hemoglobinopathy from various collection centers of Hitech Diagnostic Centre in Chennai metropolitan city. The study was conducted from year 2014–2018. Normal healthy persons were included for the control study. The study was approved by the institutional human ethics committee, Hitech Diagnostic Centre, Chennai. Written consent was taken from all the patients for using their leftover samples for research purposes. The frequency of three *HFE* gene variants C282Y, H63D, S65C was analyzed by PCR RFLP method among beta thalassemia trait (n = 203 comprising 84 males and 119 females), beta thalassemia major (n = 19 with 13 males and 6 females) and 200 age and sex-matched (87 males and 113 female cases) control samples. The effect of *HFE* variants on iron overload based on ferritin levels among beta thalassemia cases was also analyzed. Quantification of hemoglobin fractions and hemoglobin variant analysis was done by D-10 hemoglobin testing system (Bio-Rad Laboratories CA, USA) using HbA2/F/A1c dual program kit. Ferritin levels were measured to evaluate the iron status by ELISA method

PCR-RFLP technique used to identify *HFE* gene variants (C282Y, H63D & S65C). Analysis of *HFE* variants involves 1. DNA isolation 2. PCR amplification 3. Restriction enzymes digestion 4. Separation and detection of digested products through agarose gel electrophoresis. Genomic DNA was extracted using the Qiagen blood DNA extraction kit. The primer sequence for the analysis of C282Y, H63D and S65C were shown in Table 1 [31].

Table 1. Primers for *HFE* gene mutations.

Mutations	Sequence of primers 5'–3'		Fragment length
H63D	F	5'ACATGGTTAAGGCCTGTTGC3'	209 bp
	R	5'GCCACATCTGGCTTGAAATT3'	
C282Y	F	5'TGGCAAGGGTAAACAGATCC3'	400 bp
	R	5'CTCAGGCACTCCTCTCAACC3'	
S65C	F	5'ACATGGTTAAGGCCTGTTGC3'	209 bp
	R	5'GCCACATCTGGCTTGAAATT3'	

PCR was performed in veriti thermal cycler, applied biosystems. PCR was performed in 1 vial for each mutation. 12.5 µl of takkara emerald master mix, 5.5 µl dH_2O, 1 µl of forward primer, 1 µl of reverse primer and 5 µl of genomic DNA was added to a final volume of 25 µl. The PCR program for the amplification was carried out. Denaturation at 94 ℃ for 5 minutes, annealing at 63 ℃ for 1 minute. 25 cycles of extension at 72 ℃ for 1.30 minutes, again denaturation at 94 ℃ for 1 minute followed by 1 cycle annealing at 63 ℃ for 1.0 minute was done. The final extension at 72 ℃ for 3 minutes was done. PCR products with C282Y primers were digested with SNAB1 (ECO1051) Thermo Scientific #ER0401. H63D variant was digested using BCII Restriction enzyme # ERO721. S63C variant was digested with

HiNFI restriction enzyme. The digested products are electrophoresed at 1.5% agarose gel for 30–45 min. The gel is visualized under UV light. The interpretation was done based on the specific band; samples can be labeled as wild type, heterozygous or homozygous for a particular variant.

Statistical analysis

In the present study, the data were tabulated and statistically analyzed using statistical package for social sciences (SPSS Version 11.5). Student t-test, chi-square test, and proportionate tests were used to identify the significant variations and association in the study.

3. Results

The frequency and distribution of *HFE* gene variants among beta thalassemia carriers, beta thalassemia homozygous and control group were shown in Table 2. Statistical tool – pearson chi-square test was used for the analysis.

Table 2. Frequency and distribution of *HFE* gene variants among beta thalassemia carriers, beta thalassemia homozygous and controls.

S. No	*HFE* Gene Variants	BTT n = 203		BTM n = 19		Controls n = 200		Chi Square test value	Significance
1	C282Y	n	%	n	%	n	%		
	Wild	202	99.5	19	100	200	100		
	Heterozygous	1	0.5	0	-	0	-	$\chi^2 = 1.081$	NS
	Homozygous	0	-	0	-	0	-		
2	H63D								
	Wild	170	83.75	14	73.69	176	88		
	Heterozygous	33	16.25	4	21.05	24	12	$\chi^2 = 23.601$	$P < 0.01$
	Homozygous	0	-	1	5.26	0	-		
3	S65								
	Wild	203	100	19	100	200	100	-	-
	Heterozygous	-	-	-	-	-	-	-	-

Note: $P < 0.01$ Significant at 1% level; $P < 0.05$ significant at 5% level; NS: Not significant at 5% level.

Among 203 beta thalassemia trait patients studied, 202 (99.5%) cases carried wild type C282Y. One (0.5%) case was identified as heterozygous C282Y variantAmong 19 beta thalassemia major cases, all 19 (100%) cases showed wild type. The control group comprises 200 individuals, which also showed a 100% frequency of wild type C282Y. There is no significant association between C282Y *HFE* gene variant and the type of disease ($\chi^2 = 1.081$; NS). H63D genotype which is prevalent in caucasians is present among beta thalassemia carriers, thalassemia major cases and in the control group. Among 203

beta thalassemia carriers, 170 (83.75%) cases presented normal phenotype—wild type allele and 33 (16.25%) cases showed heterozygous H63D. The control group showed 176 (88%) cases wild type allele and 24 (12%) cases showed heterozygous H63D variant. The frequency was statistically significant. Among 19 beta thalassemia major cases 14 (73.69%) cases showed wild type, 4 (21.05%) cases showed heterozygous variant (H/D) and one case (5.26%) were identified for homozygous variant for H63D (D/D). Homozygous H63D (D/D) genotype was absent in beta thalassemia trait and controls. There is a significant association between H63D *HFE* gene variant and the type of disease ($\chi^2 = 23.601$; $P < 0.01$). The third *HFE* gene S65C which is rare in the Indian population is also investigated. Among 203 beta thalassemia trait, 200 control and 19 beta thalassemia major cases, all individuals were normal with wild type gene for S65C. Heterozygous and homozygous mutants were absent in the study group. In the present study, the allelic frequency of C282Y was 0.25% among beta thalassemia carriers. C282Y variant was absent in beta thalassemia major and control groups. Allelic frequency of H63D in BTT, BTM and control were 8.13, 15.8 and 6% respectively.

In the present study, *HFE* gene variants were detected using PCR-RFLP method. Figure 1 shows the detection of C282Y *HFE* variant, wild type allele is shown by the presence of 400 bp fragment and heterozygous C282Y variant showed 110 bp, 290 bp and 400 bp fragments. Figure 2 shows the detection of H63D variant. Wild type allele is marked by the presence of 187 bp fragment; heterozygous mutant for H63D shows both 187 and 209 bp fragments and homozygous H63D cases shows 209 bp fragment. Figure 3 shows the detection of S65C *HFE* variant. Only wild type allele cases were identified in the present study marked by the presence of 147 and 61 bp fragments.

Figure 1. Detection of *HFE* gene variant – C282Y by PCR-RFLP method. Lane 1: 100 bp DNA ladder; Lane 2 & 3: 400 bp fragment indicating wild type allele (WT/WT) for C282Y variant; Lane 4: 110 bp, 290 bp and 400 bp fragments indicating heterozygous C282Y/WT allele; Lane 5 to 13: 400 bp fragment indicating wild type allele (WT/WT) for C282Y variant.

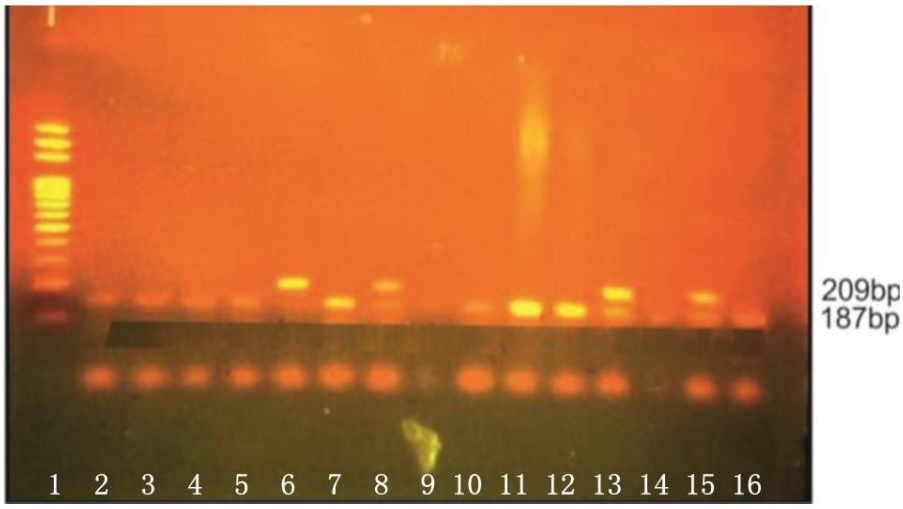

Figure 2. Detection of *HFE* gene variant – H63D by PCR-RFLP method. Lane 1: 100 bp DNA ladder; Lane 2 to 5: 187 bp fragment indicating wild type allele (WT/WT) for H63D variant; Lane 6: 209 bp fragment indicating homozygous mutant H63D/H63D allele; Lane 7, 9, 10, 11, 12, 14, 16: 187 bp fragment indicating wild type allele (WT/WT) for H63D variant; Lane 8, 13 and 15: 187 bp and 209 bp fragments indicating heterozygous mutant for H63D/WT allele.

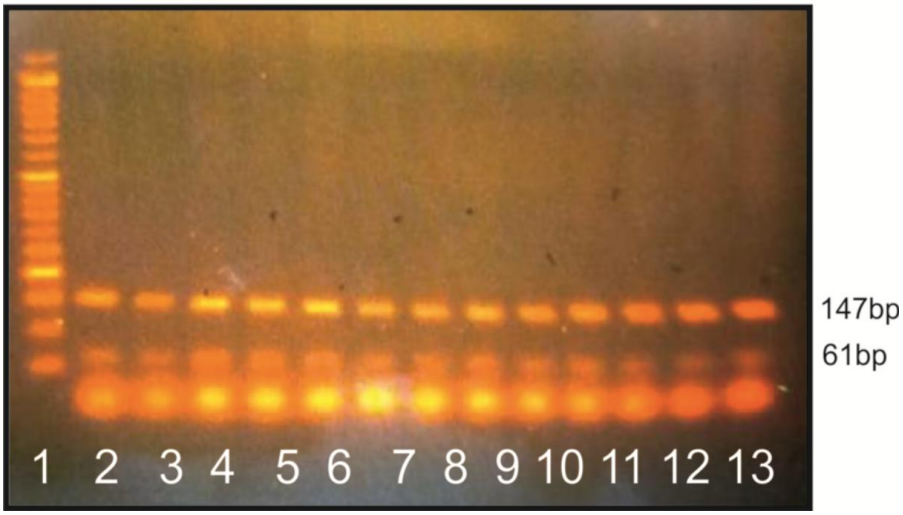

Figure 3. Detection of *HFE* gene variant – S65C by PCR RFLP method. Lane 1: 50 bp DNA ladder; Lane 2 to 15: 147 and 61 bp fragments indicating wild type allele (WT/WT) for S65C variant.

Table 3 shows the biochemical variable iron storage marker ferritin in different *HFE* genotypes among beta thalassemia trait and control group. Statistical tool – students t-test is used to compare the mean ferritin concentration among beta thalassemia trait cases and control groups with respect to *HFE* genotypes. In C282Y genotyping, 1 case with heterozygous C282Y variant among BTT has a ferritin level of 246.43 ng/ml. 202 BTT cases with wild type allele showed mean ferritin of 152.46 ng/ml and the 200 control samples with wild type allele for C282Y showed mean ferritin of 51.15 ng/ml and the values are statistically significant ($P < 0.01$).

In H63D genotype, 170 wild type H63D beta thalassemia trait cases and 176 wild type control cases showed mean ferritin of 128.65 and 48.55 ng/ml respectively. The levels are statistically significant between the groups. In both beta thalassemia trait and control group, H63D genotype with heterozygous mutant has higher ferritin levels than wild type. Similarly, the mean ferritin level of 277.97 ng/ml among 33 cases of heterozygous H63D in the beta thalassemia trait group was higher than the control groups with mean ferritin of 70.21 ng/ml have been observed. The values are statistically significant ($P < 0.01$). In S65C genotype, 203 beta thalassemia trait and 200 control samples showed wild type allele. The ferritin levels are significantly high in beta thalassemia trait categories.

Table 3. Comparison of biochemical variables of *HFE* genotypes among BTT and controls.

S. No	*HFE* Gene variants	BTT n = 203		Control n = 200		t value	Significance
		Frequency	Mean Ferritin ng/ml	Frequency	Mean Ferritin ng/ml		
1	C282Y						
	Wild Type	202	152.46	200	51.15	5.943	$P < 0.01$
	Heterozygous	1	246.43	-	-	-	-
	Homozygous	-	-	-	-	-	-
2	H63D						
	Wild Type	170	128.65	176	48.55	6.009	$P < 0.01$
	Heterozygous	33	277.97	24	70.21	2.718	$P < 0.01$
	Homozygous	-	-	-	-	-	-
3	S65						
	Wild Type	203	152.92	200	51.15	5.95	$P < 0.01$
	Heterozygous	-	-	-	-	-	-
	Homozygous	-	-	-	-	-	-

Notes: $P < 0.01$ Significant at 1% level; $P < 0.05$ significant at 5% level; NS: Not significant at 5% level.

Table 4 shows comparison of biochemical iron storage marker ferritin among beta thalassemia carrier and controls with respect to gender. Statistical tool – z test is used to compare the two proportions. Iron overload among each *HFE* genotype was classified based on biochemical iron marker ferritin. Ferritin levels >400 ng/ml among males and >200 ng/ml among females were considered as iron

overload toxicity and the results were presented. Among 203 beta thalassemia trait cases, 84 males and 119 females were analyzed for *HFE* genotyping. Wild type C282 allele was observed in 83 females and 1 case showed heterozygous variant. Among 83 wild type alleles, 73 cases had ferritin less than 400 ng/ml with mean ferritin of 89 ng/ml and 10 cases showed iron overload with mean ferritin of 588 ng/ml. In the control group, all 87 cases had normal ferritin levels which are significantly lower ($P < 0.01$) than beta thalassemia trait. In the female category, among 119 cases with wild type allele 93 cases showed low ferritin (<200 ng/ml) with a mean value of 54 ng/ml and 26 cases showed iron overload with mean ferritin of 514 ng/ml. The control group showed low ferritin levels and the results are statistically significant ($P < 0.01$). Among 84 male beta thalassemia trait cases, 12 cases with heterozygous H63D had normal ferritin levels with a mean value of 67 ng/ml and 3 cases showed iron overload with mean ferritin of 712 ng/ml. Though the control group shows lower ferritin values than beta thalassemia trait cases the values are not significant. However female H63D variant cases show statistically significant results between beta thalassemia trait and control groups.

Statistical analysis on the effect of biochemical iron storage marker ferritin among beta thalassemia carriers and controls revealed a higher frequency of iron overload among BTT with heterozygous H63D compared to controls (z score −2.56; $P < 0.05$). Among 119 female cases, 18 cases showed heterozygous variant and 101 cases were wild type allele. Eighty-two out of 101 wild type cases had ferritin <200 ng/ml and 19 cases showed iron overload with a mean value of 409 ng/ml. Seven H63D heterozygous mutant cases had high ferritin with a mean value of 802 ng/ml indicating iron overload. Three out of 15 males and 7 out of 18 females showed raised ferritin among beta thalassemia carriers. The control group had a low ferritin value among wild type and heterozygous H63D condition and the values are statistically significant ($P < 0.05$). Iron overload was absent in the control group.

Table 5 shows comparison of biochemical variables of *HFE* genotypes among BTH and controls with respect to gender. Statistical tool – z test is used to compare the two proportions. Comparison between beta thalassemia homozygous and controls revealed statistically significant results. Among 19 beta thalassemia homozygous, 13 males and 6 female cases showed wild type allele for C282Y and S65C genotype. Three out of 13 male individuals had ferritin levels <400 ng/ml, 10 cases showed ferritin levels >400 ng/ml with mean ferritin of 889 ng/ml indicating iron overload. Similarly, 6 females cases, showed wild type C282Y and S65C allele, in all these 6 cases ferritin levels were high (>200 ng/ml) with mean ferritin of 628 ng/ml. However, the control group showed normal ferritin levels. The H63D genotype showed the varied results in each genotype and 4 out of 19 male beta thalassemia homozygous cases and 11 out of 200 control cases showed heterozygous H63D. In BTM cases, 3 heterozygous H63D cases showed iron overload and the results are statistically significant (z score −3.211; $P < 0.05$). Among 11 heterozygous H63D controls, all the cases had normal ferritin levels. The results are statistically significant ($P < 0.05$). Similarly, among female beta thalassemia homozygous cases, none of the individuals showed normal levels of ferritin all the 6 cases showed high ferritin levels. One homozygous mutant for H63D observed had ferritin value of 791 ng/ml which is high than wild type *HFE* genotype. Among 113 female controls, all the samples had ferritin <200 ng/ml. The ferritin results of *HFE* genotypes between both males and females were statistically significant with ($P < 0.01$). Three male heterozygous H63D genotypes and one female homozygous H63D mutant showed ferritin of 932 and 791 ng/ml. These values are comparatively high when compared with all the *HFE* wild type genotypes indicating iron overload during co-existence of *HFE*. Iron overload was completely absent in

the control cases either in wild type allele or in heterozygous cases among all the three *HFE* genotypes. In the present study, compound heterozygous *HFE* genotypes were not observed.

Table 4. Comparison of biochemical variables of *HFE* genotypes among BTT and controls with respect to gender.

S. No	*HFE* Gene Variants	Beta Thalassemia Trait n = 203				Control n = 200				z Score	Sig.
	MALE	<400 n =	Mean ferritin	>400 n =	Mean ferritin	<400 n =	Mean ferritin	>400 n =	Mean ferritin		
1.	C282Y										
	Wild Type	73	89	10	588	87	74	0	0	−3.34	$P < 0.01$
	Heterozygous	1	246	-	-	-	-	-	-	-	-
	Homozygous										
2	H63D										
	Wild Type	62	95	7	536	76	67	0	0	−3.36	$P < 0.01$
	Heterozygous	12	67	3	712	11	120	0	0	−1.58	NS
	Homozygous	-	-	-	-	-	-	-	-	-	-
3	S65										
	Wild Type	74	91	10	588	87	74	0	0	−3.32	$P < 0.01$
	Heterozygous	-	-	-	-	-	-	-	-	-	-
	Homozygous	-	-	-	-	-	-	-	-	-	-
	FEMALE	<200 n =	Mean ferritin	>200 n =	Mean ferritin	<200 n =	Mean ferritin	>200 n =	Mean ferritin	z Score	Sig.
1	C282Y										
	Wild Type	93	54	26	514	113	34	0	0	−5.27	$P < 0.01$
	Heterozygous	-	-	-	-	-	-	-	-	-	-
	Homozygous	-	-	-	-	-	-	-	-	-	-
2	H63 D										
	Wild Type	82	54	19	409	10	35	0	0	−1.51	$P < 0.05$
	Heterozygous	11	56	7	802	13	28	0	0	−2.56	$P < 0.05$
	Homozygous	-	-	-	-	-	-	-	-	-	-
3	S65										
	Wild Type	93	54	26	514	113	34	0	0	−5.27	$P < 0.01$
	Heterozygous	-	-	-	-	-	-	-	-	-	-
	Homozygous	-	-	-	-	-	-	-	-	-	-

Notes: $P < 0.01$ Significant at 1% level; $P < 0.05$ significant at 5% level; NS: Not significant at 5% level.

Table 5. Comparison of biochemical variables of *HFE* Genotypes among BTH and controls with respect to gender.

S. No	*HFE* Gene Variants	Beta Thalassemia Homozygous n = 25				Control n = 200				z Score	Sig.
	MALE	<400 n =	Mean Ferritin	>400 n =	Mean Ferritin	<400 n =	Mean Ferritin	>400 n =	Mean Ferritin		
1	C282Y										
	Wild Type	3	199	10	889	87	74	0	0	−8.623	$P < 0.01$
	Heterozygous	-	-	-	-	-	-	-	-	-	-
	Homozygous	-	-	-	-	-	-	-	-	-	-
2	H63D										
	Wild Type	2	232	7	871	76	67	0	0	−8.026	$P < 0.01$
	Heterozygous	1	133	3	932	11	120	0	0	−3.211	$P < 0.05$
	Homozygous	-	-	-	-	-	-	-	-	-	-
3	S65										
	Wild Type	3	199	10	889	87	74	0	0	−8.623	$P < 0.01$
	Heterozygous	-	-	-	-	-	-	-	-	-	-
	Homozygous	-	-	-	-	-	-	-	-	-	-
	FEMALE	<200 n =	Mean ferritin	>200 n =	Mean ferritin	<200 n =	Mean ferritin	>200 n =	Mean ferritin		
1	C282Y n										
	Wild Type	0		6	628	113	34	0	0	−10.91	$P < 0.01$
	Heterozygous	-	-	-	-	-	-	-	-	-	-
	Homozygous	-	-	-	-	-	-	-	-	-	-
2	H63D										
	Wild Type	0	-	5	596	100	35	0	0	−10.247	$P < 0.01$
	Heterozygous	0	-	0	-	13	28	0	0	-	-
	Homozygous	0	-	1	791	0	-	-	-	-	-
3	S65										
	Wild Type	0	-	6	628	113	34	0	0	−10.91	$P < 0.01$
	Heterozygous	-	-	-	-	-	-	-	-	-	-
	Homozygous	-	-	-	-	-	-	-	-	-	-

Notes: $P < 0.01$ Significant at 1% level; $P < 0.05$ significant at 5% level; NS: Not significant at 5% level.

4. Discussion

Hereditary hemochromatosis is characterized by increased intestinal absorption and iron overload. HH is associated with *HFE* gene. In India, few studies and data pertaining to the effect of hereditary hemochromatosis on beta thalassemia have been done whereas no previous studies are available in Chennai regarding *HFE* gene variants. Hence in the present study, the frequency of *HFE* variants and their effect on iron overload among beta thalassemia carriers and beta thalassemia homozygous were evaluated.

The frequency of *HFE* genotypes is variable between different geographical regions and populations. HH is a more prevalent inherited disorder in European countries [6]. In the present study, the carrier frequency of *HFE* genotype C282Y was 0.5%, S65C was absent in the study population and H63D genotype has 16.25% carrier frequency among beta thalassemia carriers. Similarly, 21.05% and 5.26% frequency of heterozygous and homozygous H63D was noted among beta thalassemia major cases. The control group showed a lower frequency of *HFE* gene variants. In the present study, the allelic frequency of C282Y was 0.25% among beta thalassemia carriers. The C282Y variant was absent in the beta thalassemia major and control group. Allelic frequency of H63D in BTT, BTM, and control were 8.13, 15.8 and 6% respectively. Compound heterozygous *HFE* variants were absent in the study. However, in European countries, Czech Republic has reported 1.25% S65C, 3.43% C282Y and 14.97% H63D allele frequency [11]. In Spain 12.95% C282Y, 28.95% H63D and 0.69% S65C was noted [12]. In Central Europe, in the Slovenian population, the allelic frequency was 12.8% H63D, 1.8% S65C and 3.6% C282Y was reported [13]. In North-Western Europe, Netherland region, C282Y allelic frequency was around 6.0% and that of H63D was around 17% [14]. HH was pronounced more in men compared to female groups.

The frequency of C282Y and H63D is present variably among European countries. A frequency of 77.6% C282Y has been reported in Italy [15]. Similarly, the highest frequency of 28.95% H63D was reported in Spain [12]. S65C frequency was low or even absent in all continents except European countries with a prevalence ranging from 0.69 to 14.4% from Eastern, North Western, South and Central Europe regions.

In Australia, the frequency of *HFE* C282Y among BTT was 1.0% and that of H63D was 11.0% [16]. However, in African countries, Egypt has almost nil frequency of C282Y, whereas, in a study by Madani *et al*., 7.3% C282Y and 13.4% S65C have been reported among beta thalassemia carriers [17]. In North America, USA has reported 5.4% C282Y and 13.15% H63D. In Brazil, H63D frequency was higher than C282Y. S65C genotype was sub-minimal <1%.

The allelic frequency of C282Y in the present study was in line with earlier studies, reported in Egypt [9,18–20]. In a study conducted in UK, among Pakistan migrant population C282Y variants were absent [21]. In Asian countries, 1.5% C282Y among the control group was reported [22]. In India, C282Y variants was absent in earlier studies conducted in Chattisgarh, Chandigarh, Lucknow, Mumbai and Kolkata [5,23–26]. However in a study by Kaur *et al.*, C282Y variant was identified in North India, whereas it was not reported in South India [10]. Despite, 0.5% C282Y variant have been noted in the present study.

The second *HFE* variant H63D has a lower association on cell surface and regulation of iron absorption [6]. The prevalence of *HFE* H63D variant is lower than C282Y variant in European countries. However, a higher frequency of H63D has been reported in Asians and Indians [5,10]. In agreement

with the present study, an allelic frequency of 13.16% has been reported in North India [5]. H63D mutant allelic frequency of 11.0% was reported among beta thalassemic carriers in Australia [16]. In African countries, the only predominant *HFE* variant is H63D, a varying range of frequencies not less than 3% have been reported in Egypt [9,17–20,27].

In North America and South America frequency of 13–30% have been reported [28–31] In earlier studies, 8% and 8.75% H63D have been reported in Kolkata and Mumbai respectively [25,26]. The frequency distribution was in agreement with present studies. A higher frequency of H63D variants in accordance with the present study has been noted in North India [5,23,24]. In the Indian population, S65C variant was absent, similar observation has been made in the present study.

HFE gene variants involved in iron metabolism are characterized by increased iron absorption and toxicity to organs due to iron overload [2]. Similarly, beta thalassemia genetic condition is associated with varying levels of iron absorption depending on the degree of ineffective erythropoiesis which in turn leads to iron overload [32]. However in a country like India, where nutritional iron deficiency is more pronounced the impact or the complications of iron overload might be subsided or masked. In beta thalassemia major cases, the influence of blood transfusion and anemia may overlay variation in ferritin measurement. Heterozygous and homozygous H63D variant has shown higher ferritin levels than wild type and control population with statistically significant results in the present study. However, El-Rashidi *et al*., has reported H63D variantand has no profound effect on beta thalassemia patients [27]. Similarly, Fekri *et al*., has also stated that H63D polymorphism does not affect iron overload [33].

In the present study, 10 out of 33 heterozygous H63D exhibited higher ferritin levels than wild type and control group, indicating the coexistence of beta thalassemia carriers with *HFE* variants might aggravate the absorption of iron leading to iron overload. The results of the present investigation are in concordance with reports of Nadkarni *et al.* [25]. In a study conducted in East India, a higher rate of penetrance of H63D and risk of iron overload was reported in thalassemia patients [26]. Martins *et al*., has stated H63D variant will aggravate iron levels compared to wild type H63D allele [1]. In a study in Egypt, males are more prevalent to iron overload than female individuals [17]. However, in the present study, increased ferritin among heterozygous H63D was more pronounced in females than males. Females despite menstruation associated blood loss and anemia have presented with iron overload indicating the effect of *HFE* variant in heterozygous state. In a study by Soltanpour *et al*., it was stated, *HFE* H63D variant has a severe effect on iron overload among beta thalassemia cases compared to non thalassemic persons [22] the results of the present investigation is in agreement with this study. Similar results were reported by Wilson *et al.,* stating H63D variants are associated with higher ferritin levels in beta thalassemia patients [19]. Association between beta thalassemia major and H63D variant towards iron overload was reported [18]. Similar congruent results were reported by Melis *et al*., stating H63D variant has modulating effect on iron absorption and the degree of anemia induces to absorb more iron [34]. In accordance with this statement, in the present study, heterozygous H63D showed lower hemoglobin with elevated ferritin levels. Oliveria *et al*., stated thalassemia disorders and *HFE* variants will cause iron overload and insisted on the need for screening *HFE* among thalassemia patients [30]. In a study conducted in Italy, the authors have concluded that the coexistence of *HFE* and beta thalassemia trait will aggravate the risk of iron overload in beta thalassemia carriers [15]. Studies are conducted to evaluate the iron overload conditions either due to beta thalassemia or *HFE* variants, different reports have been proposed with great variations. However, monitoring iron status among beta thalassemia carriers by quantification of ferritin and hemoglobin concentration is of prime importance.

5. Conclusions

In India, few studies and data pertaining to the effect of hereditary hemochromatosis among beta thalassemia have been done whereas no previous studies are available in Chennai regarding *HFE* gene variants. The present study furnished allele frequency of H63D in BTT, BTM and controls were 8.13, 15.8 and 6% respectively. Ten out of 33 heterozygous H63D exhibited iron overload with higher ferritin levels than wild type and control group, indicating the coexistence of beta thalassemia carriers with *HFE* variant. *HFE* variants might aggravate the absorption of iron leading to iron overload. Hence it is inferred from the present investigation, even so beta thalassemia has a selective advantage over iron absorption, the presence of *HFE* genotypes either in heterozygous state or homozygous condition aggravates iron absorption by elevating ferritin levels. Analysis of *HFE* genes and iron status will remarkably help to reason out the probable reason behind the iron status and support in proper management of beta thalassemia cases. Management of beta thalassemia cases with iron supplementation for iron-deficient beta thalassemia cases or chelators to remove the iron toxicity will help the quality of life in these individuals.

Acknowledgments

The authors thank HOD of Zoology, Principal, Pachaiyappa's college, Chennai, for their support. The authors are very grateful to the authorities of Hitech Diagnostic Centre, Kilpauk, Chennai for their support and valuable suggestions.

Conflict of interest

Authors declare no conflict of interest in this manuscript.

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