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Research Article

MUTATIONAL ANALYSIS OF HFE GENE IN PREMENOPAUSAL AND MENOPAUSAL WOMEN IN POPULATION OF GUJARAT

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ABSTRACT
Heriditary hemachromatosis(HH) is the prototype disease for primary iron overload. The gene that causes most cases of HH is designated as HFE. Two missense mutations (C282Yand H63D) of this gene are found to be associated with HH phenotype. In present study, our aim is to find out HFE gene mutations in premenopausal and menopausal women in population of Gujarat.
Polymerase chain reaction-restriction fragment length polymorphism method was used for screening C282Yand H63D mutation in 54 subjects [Premenopausal phase iron-overload subjects (27); Menopausal phase iron-overload subjects (27)] and 21 healthy controls.
Our findings show that out of 54 female iron-overload subjects, 1 had a heterozygous mutation of the H63D region of HFE gene. The genotype frequency of menopausal phase female subjects was 24(89 %) for homozygous H63D and 3(11.11%) for heterozygous H63D. This corresponds to allelic frequency of 94.44% for C-allele. The second important finding of our study was that all subjects were free from C282Y mutation.
The results of our case-control study indicate that H63D has a positive association with iron-overload. We did not find any C282Y mutation among the women who participated in this study. Ideally, a sample larger than ours should be studied in a genetic association study to rule out the chance factor.

Key Words: *HFE gene, Primary Iron Overload, Heriditary Hemachromatosis, HFE Mutations*

INTRODUCTION
Iron is indispensable for basic cellular functions. However, this metal is also a catalyst for chemical reactions associated with the production of reactive oxygen species, which may lead to oxidative stress and cellular damage. Hence, the controlled regulation of iron homeostasis is necessary to keep the body iron at a moderate level to avoid iron deficiency and iron overload (Parkkila *et al*., 2001). Body iron homeostasis is regulated primarily by duodenal and upper small intestinal absorption and is responsive to body iron stores. Hence, iron absorption is increased during iron deficiency and down-regulated when iron are replete (Pietrangelo *et al*., 1995). There is no effective method for the elimination of excess body iron, and iron overload from iatrogenic or idiopathic pathological causes can lead to multiple systemic complications. Hereditary haemochromatosis, the prototype disorder of iron overload due to misregulated iron homeostasis in humans, is caused by an inappropriate increase in iron absorption in the duodenum and upper small intestine. Iron overload increases the risk of various clinical complications such as arthritis, diabetes, cardiomyopathy, skin pigmentation, gonadal failure and liver cirrhosis (CLD) (Fleming *et al*., 2002).
The genes identified to be responsible for primary iron overload in HH are HFE, HJV, HAMP, TFR2 and SLC11A (Feder *et al*., 1996). The hereditary hemochromatosis gene *HFE* plays a pivotal role in iron homeostasis. The hereditary hemochromatosis gene *HFE* (6p21.3), 4 Mb telomeric to the *HLA-A* locus, and its product has a structure similar to MHC class I molecules. It has a critical role in iron homeostasis. Two missense mutations (C282Yand H63D) of this gene are found to be associated with HH phenotype (Feder *et al*., 1996). The C282Y mutation, most frequent amongst Caucasians (Beutler *et al*., 1996) results from a G-to-A transition at nucleotide 845 of the HFE gene (845 G → A) that produces a substitution of cysteine for a tyrosine at amino acid position 282 (C282Y) in the protein product. In the

H63D mutation, a C-to-G transversion at nucleotide 187 of the gene (187 C → G), results in a substitution of histidine for an aspartate at aminoacid position 63 (H63D) in the HFE protein. In addition to C282Y and H63D, nine other missense mutations causing amino acid substitutions have been documented. In one, a substitution of a cysteine for serine at amino acid position 65 (S65C) has been implicated in a mild form of HHC (Mura *et al*., 1999). A number of intronic polymorphisms have also been found (Pointon *et al*., 2000).

The *HFE* protein is a 343 residue type I transmembrane protein that associates with class I light chain beta2- microglobulin (Feder *et al*., 1996). The *HFE* protein product binds to the transferrin receptor, forms a stable complex with transferrin receptor (TFR), and thereby reduces its affinity for iron-loaded transferrin by 5- to 10-fold (Feder *et al*., 1998). TF is the major iron transport protein in blood and TFR facilitates the uptake of ironbound transferrin. The C282Y mutation prevents the association of the mutant HFE protein with TFR, disrupting its transport to and presentation on the cell surface (Lebron *et al*., 1998). As a result, increased affinity of the uncomplexed TFR for TF causes higher iron absorption. The H63D mutation, in contrast, does not appear to prevent beta2-microglobulin association or cell surface expression (Waheed *et al*., 1999). Therefore, the significance of the H63D mutation on the *HFE* gene was initially controversial (Penny at al., 1998). Subsequent studies suggested a functional role for H63D mutation (Beutler *et al*., 1997; Fairbanks *et al*., 1998). The H63D variant of the HFE protein does reach the cell surface and forms a stable complex with TFR but fails to control high TFR affinity for TF (Waheed *et al*., 1997). The localization of the *HFE* protein in the crypt cells of the duodenum (the site of dietary iron absorption) and its association with transferrin receptor in those cells are consistent with a role in regulating iron absorption (Waheed *et al*., 1999; Parkilla *et al*., 1997). The observation that *HFE*-deficient mice (*HFE* gene knockout model) develop iron overload similar to that seen in human HHC provides evidence that the *HFE* protein is involved in regulating iron homeostasis (Zhou *et al*., 1998).

MATERIALS AND METHODS

The blood samples were collected from 75 different subjects and categorized in different groups like: Group I- Control(21); Group II- Premenopausal phase iron-overload subjects(27); Group III- Menopausal phase iron-overload subjects(27). These three groups of subjects were tested for the two mutations.

Genomic DNA was isolated from the peripheral blood leukocytes by modified method (Boretto *et al*., 1992) using whole blood. The C282Y and H63D loci were analyzed in controls and patients in order to confirm the two mutations. The C282Y and H63D mutations were then screened using enzymatic digestion of PCR products encompassing the mutation sites. As the C282Y mutation creates a new *Rsa*I restriction site, the 387-bp PCR product digested with *Rsa*I shows two fragments of 247 and 140 bp in normal DNA while three fragments of 247, 111, and 29 bp are generated in mutated DNA. The H63D mutation destroys a *Mbo*I site in the 208-bp PCR product, while normal DNA is cut into two fragments of 138 and 70 bp. PCR reactions were carried out in a total volume of 50 ml containing 500 ng of genomic DNA, 30 pmol of each primer, 5 m*M* MgCl2, 80 m*M* TRIS-HCl pH 9, 20 m*M* (NH4)2SO4, 200 m*M* dNTP, and 1.25 U *Taq* polymerase. Amplification was performed by 30 cycles each consisting of denaturation for 1 min at 94° C, annealing for 1 min at 54° C, and extension for 1 min at 72° C. Amplification primers were as follows: C282Y, forward primer 5'-TGGCAAGGGTAAACAGATCC-3', reverse primer 5'-CTCAGGCACTCCTCTCAACC-3'; H63D, forward primer 5'-ACATGGTTAAGGCCTGTTGC-3', reverse primer 5'-GCCACATCTGGCTTGAAATT-3'. Restriction reactions were carried out for 3 h at 37° C using 15 ml of the PCR product and 5 U of enzyme. The restriction fragments were electrophoresed in 3% Agarose gels stained by ethidium bromide.

RESULTS AND DISCUSSION

In present study, we have analyzed HFE gene mutations (C282Y and H63D) in different subgroups of female iron overload subjects and controls to find a relationship between iron overload and HFE mutation in the population of Gujarat. Aim of our study was to investigate the role of HFE gene mutations in iron-

overload female subjects. Endogenous estrogens are more closely related to menopausal phase and, (Liehr *et al*., 2001) iron might have a role at redox-cycling estrogen metabolites to produce hydroxyl radicals (Syrjakoski *et al*., 2006), hence we studied menstrual and menopausal iron-overload subjects for the HFE H63D and C282Y mutation. Since significant iron loss stops with menopause, an increase in HFE-associated hemachromatosis risk might also be possible. Transferrin saturation of 40-60 or serum ferritin of 200-300 ng/mL (females) indicates iron-overload and transferrin saturation of >60 or serum ferritin of >350 ng/ml indicates primary hemachromatosis.

The two mutations were searched in 54 carefully selected iron-overload menstrual phase and menopausal phase female subjects and 21 normal female controls. Our findings show that out of 54 female iron-overload subjects, 1 had a heterozygous mutation of the H63D region of HFE gene. The genotype frequency of menopausal phase female subjects was 24(89 %) for homozygous H63D and 3(11.11%) for heterozygous H63D. This corresponds to allelic frequency of 94.44% for C-allele. The proportions of patients with iron-overload and healthy volunteers who carried mutant allele are presented with 95% confidence intervals (CIs) and Odds Ratio (OR).

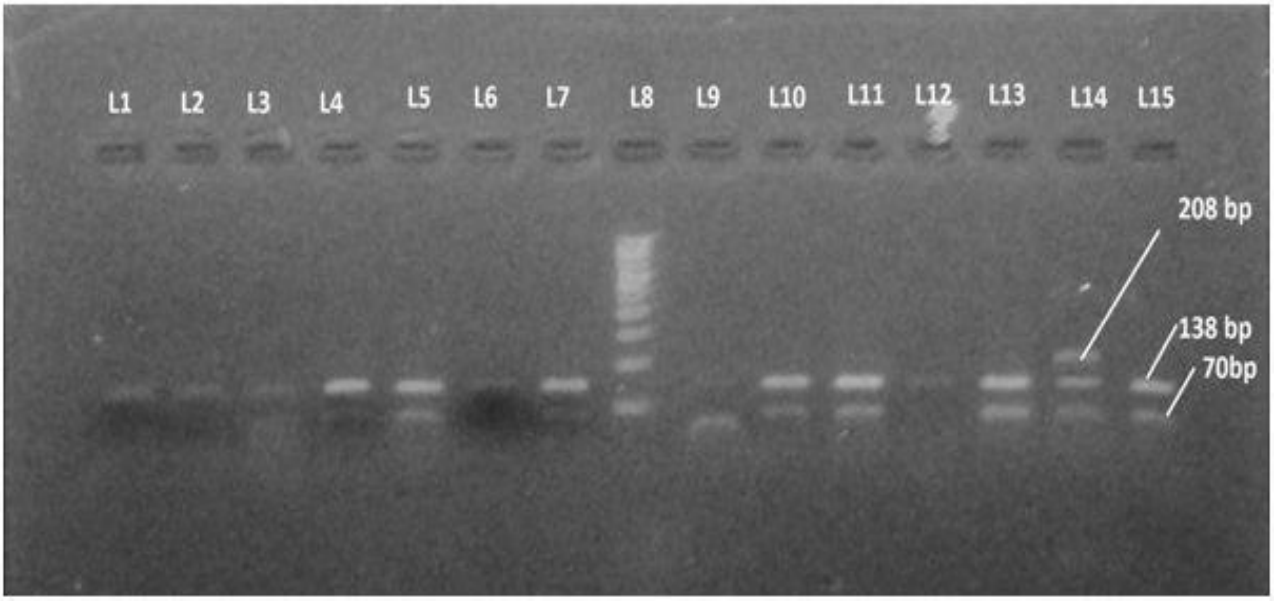

Figure 1: Determination of H63D mutation of the HFE gene by restriction enzyme analysis

Amplified product of HFE gene was digested with MboI restriction enzyme and resolved in 2% gel. In figure, L1-L4 represents Group I, L5-L7 & L9 represents Group II and L10-L15 represents Group III. L8 shows 100 bp ladder.

The second important finding of our study was that all subjects were free from C282Y mutation. The results of this case-control study showed that H63D mutation was more common in iron-overload subjects than C282Y mutation. The genetic mutation and biochemical iron overload state did not therefore seem to be related in the majority.

Amplified product of HFE gene was digested with Rsa I restriction enzyme and resolved in 2% gel. In figure 12, L1-L4 represents Group I, L5, L6, L8 & L9 represents Group II and L10-L12 represents Group III. L7 shows 100 bp ladder.

A study was performed by Barton and co-workers (Barton *et al*., 2004) in which they showed a significant association between female breast cancer and HFE-H63D, that also revealed a non-significantly increased odds ratio for H63D carriers (n =18, OR = 2.0, *P* = 0.14) in breast cancer. A related article to Barton's study made the same comparisons of H63D mutation frequency between

Russian women with breast cancer and controls and found age to be an important confounder of the association, wherein a positive association of H63D with breast cancer was only found among women

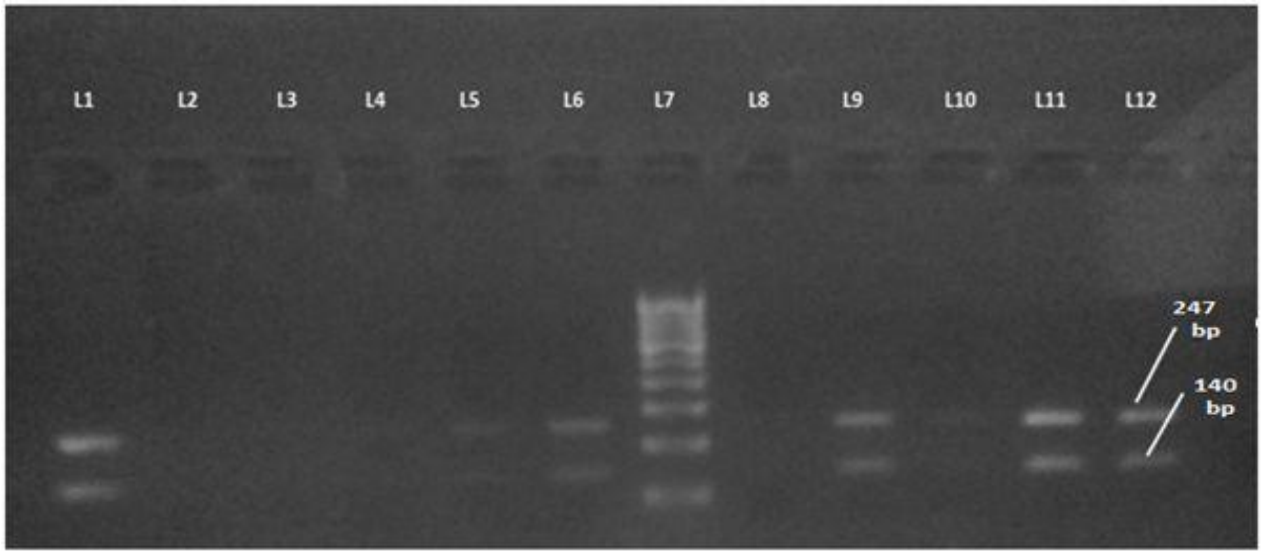

Figure 2: Determination of H63D mutation of the HFE gene by restriction enzyme analysis

over 57 years old (Kondrashova *et al*., 2005). A study showed no association between male breast cancer and H63D. Although the lack of gender difference shown in association between *HFE* genotypes and medical conditions related to iron overload other than cancer (Adams *et al*., 2005), this variability in women with H63D mutations with respect to cancer risk could be the influence of potential modifier factors for breast cancer. The only other cancer association with H63D is with malignant gliomas (Tavazzi *et al*., 2001).

Our data was well supported by a recent study, wherein Shalu Jain and co-workers analyzed the serum iron parameters and HFE gene mutations (C282Y and H63D) in different subgroups of cirrhosis patients and controls to find a relationship between iron overload and HFE mutation in Indian population. According to their findings, only 2 of 13 patients of a particular subtype of liver cirrhosis had a mutation of the HFE gene. None of these patients however had evidence of hemochromatosis. The genetic mutation and biochemical iron overload state did not therefore seem to be related in the majority. Primary hemochromatosis appears rare in Indian patients, as they failed to find any in 496 consective patients with liver cirrhosis.

The second important finding of their study was the low frequency of the mutated C282Y allele in Indian subjects. Most of the earlier data, except a couple of studies (Kaur *et al*., 2003) showed complete absence of C282Y mutation in Indian population (Garewal *et al*., 2005; Dhillon *et al*., 2007; Thakur *et al*., 2004). C282Y mutation is the commonest one described in patients with hemochromatosis in people of European descent. Its very low frequency in Indian subjects may explain the paucity of this disease in this country, and underscores the genetic differences between populations. In contrast to C282Y mutation, H63D was more common in most of studies from India (Shukla *et al*., 2006).

Our study had some limitations. We were unable to study the effect of H63D and C282Y on serum iron parameters or gene and environment interactions, as we did not have stored serum samples from patients nor dietary or medication (oral contraceptives, etc) history. Ideally, a sample larger than ours should be studied in a genetic association study to rule out the chance factor. Not only is the study small, but when a small number of multiple comparisons are made, the power of the study is further diminished.

Conclusion

The results of our case-control study indicate that H63D has a positive association with iron-overload. We did not find any C282Y mutation among the women who participated in this study. However, when a

small number of multiple comparisons are made, the power of the study is diminished. Ideally, a sample larger than ours should be studied in a genetic association study to rule out the chance factor.

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