Variable phenotypic presentation of iron overload in H63D homozygotes: are genetic modifiers the cause?

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Abstract

Background—First considered as a polymorphism of the HFE gene, the H63D mutation is now widely recognised as a haemochromatosis associated allele. But few H63D homozygotes with clinical manifestations of hereditary haemochromatosis (HH) have been reported. Concurrently, an increasing number of genes have been shown to interact with HFE in iron metabolism.

Aims—To describe the clinical expression of iron overload (IO) associated with H63D homozygosity, and search for potential genetic modifiers (within the HFE or other genes) that could explain the variability of the phenotypes.

Patients and methods—We retrospectively analysed the clinical phenotype of 56 H63D homozygotes referred for a personal or family history of IO. For each subject we examined intragenic HFE haplotypes and transferrin receptor (TfR) gene polymorphisms and searched for the Y250X mutation on the TFR2 gene. Additionally, we sequenced the HFE gene of H63D homozygotes with HH.

Results—Fifty of 56 subjects had biological and/or clinical abnormalities of iron metabolism. Up to two thirds of patients (n=34) had no acquired cause of IO. Among these, 12 had a phenotypic diagnosis of HH. In the iron loaded group there was a strong prevalence of male patients. No correlation was found between the potential genetic modifiers and phenotypes. No additional mutation of HFE was identified.

Conclusion—The variable phenotypes associated with H63D homozygosity do not appear to be linked to other HFE mutations, to the TFR2 Y250X mutation, or to HFE or TfR gene intragenic polymorphisms. The exact role of H63D homozygosity in IO and HH needs to be further investigated in unselected populations.

Keywords: haemochromatosis; H63D homozygotes; phenotypic variability; HFE haplotypes; transferrin receptor gene

The most common genotype associated with clinical manifestations of hereditary haemochromatosis (HH) corresponds to the ancestral C282Y mutation at the homozygous state on the HFE gene identified by Feder et al in 1996. The recently published EASL consensus conference on haemochromatosis offers more up to date knowledge of the disease. While C282Y homozygosity accounts for more than 80% of HH in most countries worldwide with a predominantly Northern European population, other HFE genotypes are associated with a typical presentation of HH among which are the compound heterozygous state for C282Y and the H63D mutation, and a few private mutations. The significance of the H63D mutation on the HFE gene was first controversial. Feder et al suggested it could be either a causative mutation or a polymorphism; a few authors then considered it as a simple polymorphism. The implication of the H63D mutation as a deleterious event on the HFE gene was subsequently established by clinical and molecular studies. Firstly, an excess of H63D alleles was clearly demonstrated among HH patients. Then, compound heterozygotes for the C282Y and H63D mutations were found to express HH, even though with less penetrance than C282Y homozygotes. In 1998, in vitro evidence for the functional consequences of the H63D mutation in HFE protein activity was reported. The causative role of the H63D mutation in HH or iron overload (IO) is now established but the question of the penetrance of this allele is still unclear. Variability in phenotypic expression is observed in many monogenic diseases, and HFE related HH is no exception. Asymptomatic C282Y homozygotes have been reported and the penetrance of the compound heterozygous state for C282Y and H63D seems to be incomplete. Moreover, clinical expression of IO in H63D homozygotes is still a matter of debate and represents a recurring question for physicians who see HH patients. However, to our knowledge, comprehensive data on a large cohort of such homozygotes have not been reported. To further investigate the relationship between H63D homozygosity and IO, we studied the clinical and genetic characteristics of 56 probands with this genotype.

Abbreviations used in this paper: HH, hereditary haemochromatosis; IO, iron overload; Tf, transferrin; TfR, transferrin receptor; TFR2, transferrin receptor 2; HH, hepatic iron index; OR, odds ratio; DIOS, dysmetabolic iron overload syndrome; PCT, porphyria cutanea tarda.
As variable clinical penetrance of a given allele can be due to genetic modifiers, we tested a number of genetic factors which could be involved in the variable phenotypic expression associated with H63D homozygosity. Genetic modifiers that may explain such a variability can be intragenic polymorphic sequences, extrinsic genetic factors such as other genes implicated in the same metabolic pathway, or environmental factors. Here we have analysed intragenic haplotypes and searched for new mutations in the HFE gene. In addition, we tested the hypothesis that transferrin receptor (TfR) varieties could influence HFE protein behaviour by studying intragenic polymorphisms of the TfR gene. Finally, we searched for the Y250X mutation on the transferrin receptor 2 (TFR2) gene which was recently implicated in non-HFE linked haemochromatosis.

Patients and methods

Patients

The study was carried out on 968 consecutive samples received in our laboratory for HFE genotyping. Reasons for the search for HFE mutations were: (1) biological and/or clinical suspicion of HH; (2) family screening for HH; or (3) known diagnosis of HH. C282Y and H63D mutations were systematically screened in all subjects. Blood samples were obtained after informed written consent. Overall, 60 individuals were H63D homozygotes. Clinical, biological, and histological parameters were evaluated for each subject as well as treatments. A questionnaire was established and completed using the medical records of the patients. The parameters studied were: age at the moment of DNA analysis; sex; indication for the search for HFE mutations; and personal and family history (including HH, IO, diabetes, hepatic disease, and cancer). Clinical criteria for HH were collected, among which were fatigue, diabetes, endocrinopathy, cardiopathy, hepatopathy, arthropathy, and skin lesions. Additionally, we calculated body mass index and looked for the presence of high blood pressure. The following biological parameters were assessed: serum iron, transferrin saturation, serum ferritin, glycaemia, cholesterol, and triglycerides. Blood cell count and viral hepatitis status were examined in all patients. When a liver biopsy had been performed, we recorded the presence of fibrosis, cirrhosis, hepatic iron concentration, and hepatic iron index (HII). Finally, when a depleting treatment had been performed, we noted the number, volume, and periodicity of venesections to calculate total body iron removed. The criteria for HH have been reviewed in light of the recently published EASL consensus conference on haemochromatosis.

Methods

Diagnosis of C282Y and H63D mutations

Both HFE mutations were investigated using a method previously described, based on modification of natural restriction sites.

Table 1. HFE genotypes of the whole population referred for a personal or family history of iron overload (patients) compared with a control group (controls) from our area (1276 unselected newborns)

<table>
<thead>
<tr>
<th>HFE genotype</th>
<th>Patients</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>m1m1</td>
<td>178</td>
<td>2</td>
</tr>
<tr>
<td>m1N</td>
<td>141</td>
<td>58</td>
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<tr>
<td>m1m2</td>
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<td>170</td>
<td>345</td>
</tr>
<tr>
<td>NN</td>
<td>334</td>
<td>820</td>
</tr>
<tr>
<td>Total</td>
<td>968</td>
<td>1276</td>
</tr>
</tbody>
</table>

Results

Clinical data

HFE genotypes of the 968 tested subjects are given in table 1. The frequency of H63D homozygotes among patients was significantly higher than in a control sample of 1276 unselected newborns in our area (p=10^-18, OR 2.3 (95% CI 1.5–3.5)).

Of the 60 identified H63D homozygotes, full data were obtained for 56 who were then included in the study. There were 42 males (75%) and 14 females (25%). Mean age was 53.8 (14) years. Reasons for the search for HFE mutations in these H63D homozygotes are listed in table 2.
Only six H63D homozygotes in this cohort had no biological signs of IO. Mean age of this group was lower (42.3 (21.5) years) than that of iron loaded subjects (54.7 (12) years) but the difference was not statistically significant (p=0.06). The number of men and women was the same in both groups. All six were diagnosed through family screening.

Fifty of 56 H63D homozygotes (89.3%) had biological and/or clinical abnormalities of iron metabolism (fig 1): 39 men and 11 women (sex ratio males to females 3.5). An acquired cause of IO was searched among the iron loaded H63D homozygotes. Nine were habitual drinkers (that is, consuming more than 30 g alcohol/day) and one had chronic viral hepatitis B. Three of these 50 H63D homozygotes (6%; two males and one female aged 38, 54, and 70 years, respectively) fulfilled the diagnosis criteria for dysmetabolic iron overload syndrome (DIOS). As described, transferrin saturation was normal and serum ferritin levels were elevated (557 µg/l on average). Both males had a body mass index greater than 25 and both had hyperlipaemia. The woman had a normal body mass index and no dyslipidaemia but she had high blood pressure. In all three cases liver biopsy showed a HII lower than 1.9.

A diagnosis of porphyria cutanea tarda (PCT) was established in four of 50 iron loaded H63D homozygotes (8%) based on clinical and biological criteria. This group comprised one family and three sporadic cases. All subjects with PCT were males and were undergoing phlebotomies. Two had excessive alcohol consumption while one had hepatitis C virus related hepatitis. No cause of IO was found in the fourth subject. Liver biopsy performed in two cases showed chronic hepatitis in the hepatitis C virus patient and a HII <1.9 in the second.

Thus among 50 H63D iron loaded subjects, 16 (32%) had a secondary cause of IO. Conversely, for 34 of 50 (68%) H63D homozygotes, abnormalities of iron metabolism were not associated with a known aetiology. One was the fourth patient with PCT. Twelve of these patients had a phenotypic diagnosis of HH (table 3). The sex ratio (male/female) was 5 and mean age at diagnosis was 53.4 (14) years (range 35–77). Eleven of these 12 individuals had a liver biopsy. HII was greater than 2 in eight subjects; it was less than 1.9 in one subject and was not determined in two. Fibrosis was present in six of 11 liver biopsies; three of these six also showed cirrhosis. A histologically proved hepatocarcinoma with cirrhosis was identified in two patients who died shortly after diagnosis. Mean mobilised body iron was 7.3 (7.6) g (range 1.8–27), calculated for all patients except those with hepatocarcinoma. Finally, four of 12 HH subjects had a family history of HH and two had a family history of hepatocarcinoma.

Among the 22 remaining individuals, abnormalities of iron metabolism had a variable expression and were not associated with a well defined pathology. A family history of HH or IO was found in five of 22 cases (23%). The sex ratio of this group of 22 subjects was 1.6 and mean age was 54.1 (11.6) (range 35–77). Fatigue and arthritis were the two most frequent clinical symptoms (40%). Mean serum ferritin level was 824 µg/l (range 26–1715) and mean transferrin saturation was 57.8% (range 29–96). Five had a liver biopsy (HII was <1.9 in two and not determined in three). One individual who had a liver biopsy was diagnosed with a hepatocarcinoma but hepatic iron was not assessed. Four subjects had been occasionally phlebotomised (<5 g iron removed). Comparisons of mean age, mean serum ferritinaemia, and mean transferrin saturation between males and females showed no significant differences. Accordingly, in these 22 subjects abnormalities of iron metabolism could not be related to any known aetiology, the only common feature being H63D homozygosity associated with hereditary IO in 25%.

**HAPLOTYPE ANALYSIS OF THE HFE GENE**

A single intragenic HFE haplotype (IVS2 (+4) c/c, IVS4 (+907) a/a) was observed among the 56 H63D homozygotes, as described previously. Some of the patients’ haplotypes included in the present study have been reported in part elsewhere.

**SEQUENCE ANALYSIS OF THE HFE GENE**

All H63D homozygous patients with a phenotypic diagnosis of HH were submitted to DNA sequencing of the HFE gene. No sequence
changes were found apart from the above reported polymorphisms.

GENETIC POLYMORPHISM OF THE TFR GENE
The TFR Ser142Gly polymorphism was examined for all 56 H63D homozygotes. No correlation was found between any of the TFR alleles or in the corresponding TFR genotypes, and the clinical phenotypes of the 56 subjects. In particular, the Ser142 allele which has been found to be associated with carcinogenesis in association with the C282Y mutation21,22 was not found to be linked with severe manifestations in H63D homozygotes. Moreover, the four H63D homozygous patients with hepatocarcinoma had no specific TIR allele or genotype.

CHARACTERISATION OF THE TFR2 Y250X MUTATION
All 56 H63D homozygotes were investigated for the presence of the Y250X mutation on the TFR2 gene. None was found. Some of these results concerning the 12 H63D homozygotes with a phenotype of HH have been reported elsewhere as part of a larger series of non-C282Y HH patients (Aguilar-Martinez, personal communication).

Discussion
CLINICAL VARIETY OF IO ASSOCIATED WITH H63D HOMOZYGOSITY

The allelic frequency of the H63D mutation is very variable worldwide.23 In the general population in our area it is 17%, and the frequency of H63D homozygotes reaches 3%,17,18 This high prevalence can be explained by our geographical position in southern France. The population of this area is heterogeneous, comprising people originating from various Mediterranean countries, including Spain, where the H63D mutation has the highest frequency in the world.19,20 However, the frequency of the H63D homozygous genotype was significantly higher (p<0.0001) among patients referred for a personal or family history of IO than in the general population of the area. One explanation could be that if H63D homozygosity leads to different degrees of IO due to a variable penetrance of this genotype, a higher number of H63D homozygotes in the general population would allow more iron loaded H63D homozygotes to be found than in studies on Northern European populations. Indeed, in previously described populations from the USA, Canada, and Australia19-26 the H63D mutation was less frequent, resulting in less iron loaded H63D homozygotes being diagnosed. Here, we investigated a group of 56 H63D homozygotes. Their clinical presentation varied considerably; only six were recruited through family screening not exhibiting IO.

ACQUIRED IO IS FOUND IN LESS THAN ONE THIRD OF H63D HOMOZYGOTES

All subjects investigated in this study were carefully screened for secondary causes of IO. Excessive alcohol intake and viral hepatitis were found in 13 cases (26%). Three of these had clinical manifestations of PCT (two with excessive alcohol consumption and one with hepatitis C virus). The worsening role of IO in PCT is well known in both sporadic and family forms, and a high prevalence of HFE gene mutations has been demonstrated in PCT.30,31 The prevalence of the C282Y mutation in PCT seems to be higher in subjects of Northern European descent than in individuals from southern countries.32,33 This could be because of the respective distribution of HFE mutations in both populations. Thus HFE genotypes that favour iron accumulation seem to contribute to the development of PCT with a gradient locating C282Y in the north of Europe and H63D in the south.32,33 Three additional patients fulfilled the criteria for DIOS,30 DIOS has been described as a disease distinct from HH. However, the prevalence of HFE gene mutations in this syndrome is high (60%),34 and some authors suggested that DIOS could be a clinical form of HH.35 Recently, other groups have argued for a unique origin of DIOS and HFE related HH.36,37 NEARLY 70% OF H63D HOMOZYGOTES HAD NO DETECTABLE CAUSE OF IO

In these 34 subjects, IO can be severe or mild. Severe IO was observed in nearly 25% of the 50 iron loaded subjects; 12 patients presented a phenotypic diagnosis of HH. In our centre, we...
have previously shown that severe IO is mostly due to homozygosity for the C282Y mutation (81.8%). In this preliminary series of 99 patients, compound heterozygotes accounted for 7.1% of HH cases while H63D homozygotes represented 4% of the total. The non-HFE linked HH phenotype was present in only 3% of this series. The phenotypic expression of HH in the 12 H63D homozygotes was not different from C282Y homozygotes except for the sex ratio. The sex ratio (male/female) was 5 in H63D homozygotes manifesting HH whereas it was only 1.5 in our sample of C282Y homozygotes (data not shown). A high prevalence of males among subjects with so-called “mild HFE genotypes” (H63D, S65C) with clinical manifestations has been observed previously. Other parameters such as age of occurrence of the disease, clinical, biological and histological manifestations, as well as removed iron were not different in H63D homozygotes with HH and those usually reported in HH patients. It is noteworthy that two of 12 (16.6%) homozygous H63D HH patients had hepatocarcinoma. Such a high prevalence of hepatocarcinoma is not observed in classical haemochromatosis and a normal prevalence of the C282Y mutation has been reported in patients with hepatocarcinoma. In this last work the H63D mutation was not investigated. Because of the small number of affected subjects in our series, further studies are needed to evaluate the relationship between H63D and hepatocarcinoma.

Iron overload of milder severity (minimal or modest according to the definition of the EASL consensus conference) was present in the remaining 22 individuals. Compared with the group of H63D patients with HH, mean age was not different (54.1 ± 33.4 years) whereas the sex ratio was lower (1.6 ± 5 in H63D homozygotes with HH). The higher number of females in this group could explain the weaker expression of HH in H63D homozygotes. However, as we have not screened the full length genomic sequence of HFE, we cannot exclude the fact that a modifier, located on deep intronic sequences or in the 5' or 3' distant regions, could be involved.

The transferrin receptor (TfR) gene is one of the main candidate genes that could participate in modifying HFE activity. The HFE protein has been demonstrated to regulate transferrin receptor binding with transferrin (Tf) in vitro. In normal subjects, the HFE protein has been shown to bind to TfR on the cell membrane and lower its affinity for Tf. Both HFE mutant proteins, C282Y and H63D, display an impaired inhibiting effect on TfR binding to Tf. More recent data have confirmed the inhibitory effect of HFE on TfR but seem to indicate that it could be related to the intracellular biosynthesis pathway of both proteins. Moreover, the H63D mutation is located on the HFE α1 domain and it is noteworthy that the TfR binding site on HFE has been shown to be located on α1 domain of TfR and adjacent loop. TfR mutations have been investigated in patients with HH in the absence of the C282Y mutation, either in the homozygous or compound heterozygous state. This study failed to find any deleterious sequence alteration on the TfR gene but identified a small number of previously described TfR polymorphisms. Concurrently, a relationship between specific TfR polymorphisms, the C282Y mutation, and carcinogenesis has been reported. The authors suggested that this could reflect an enhanced IO process in relation to HFE mutants. New described animal models strengthen the hypothesis of a role for TfR to explain non-HFE related HH phenotypes. In the present study we failed to find any correlation between the clinical severity of IO in H63D homozygotes and a specific TfR allele, even in subjects with hepatocarcinoma. However, we have not tested the whole TfR gene sequence implicated in the relationship with HFE. More recently, a mutation (Y250X) on a homologous protein to TfR, TFR2, has been suggested to be involved in severe IO in humans. Moreover, one of the patients with this mutation was also a H63D homozygote. This association could suggest that the Y250X mutation on the TFR2 gene is
H63D homozygotes

In fact, none of the 56 H63D homozygotes of our sample had the Y250X mutation. Thus our results do not support the fact that this mutation could be the common mechanism underlying IO in H63D homozygotes in our area. It seems highly probable that the Y250X mutation, which has been found in two unrelated Sicilian families, could be a private mutation in this island population. Its association with H63D homozygosity, which is more widespread, was certainly fortuitous.

NON-GENETIC FACTORS COULD MODIFY THE PHENOTYPIC EXPRESSION OF H63D HOMOZYGOSITY

The genetic modifiers investigated in the present study did not appear to be implicated in the variable phenotypic expression of H63D homozygotes. In contrast, some non-genetic factors could be involved such as age and sex. Firstly, mean age in the group of H63D homozygotes who did not express any sign of IO was lower than in the group of individuals with IO (42.5 ± 54.7 years). Secondly, there was a high prevalence of males in the iron loaded groups, especially among those with the severe phenotypes. These age and sex influences need to be confirmed in additional H63D homozygotes.

Conclusion

In a group of 56 H63D homozygotes, recruited on the basis of the presence of personal or family disorders of iron metabolism, we found a wide variety of phenotypes, from normal individuals to patients with severe IO. The search for genetic modifiers in the HFE, TJR, and TFR2 genes was negative. However, other as yet unknown genetic or non-genetic modulating factors could be involved. A prospective study on non-selected H63D homozygote adults from the general population may help reach definitive conclusions. Nevertheless, when a diagnosis of H63D homozygosity is made, it seems to warrant a clinical and biological follow up, particularly in men, as some of these individuals can develop severe phenotypes, including haemochromatosis.

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