

Compound Heterozygotes for Hemochromatosis Gene Mutations: May they Help to Understand the Pathophysiology of the Disease?

Submitted 07/07/97; revised 07/11/97

(communicated by Ernest Beutler, M.D., 07/11/97)

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ABSTRACT: Two mutations have been described on the gene considered to be responsible for genetic hemochromatosis, the HLA-H or *HFE* gene. The C282Y mutation is a disease-causing mutation in most cases of genetic hemochromatosis, but involvement of the H63D substitution in the pathogenesis of the disease is unclear. Compound heterozygotes for both substitutions could help to determine whether or not the second mutation is a worsening factor when associated in *trans* with the C282Y mutant. We found twenty nine compound heterozygotes during DNA analysis of patients referred to our laboratory for the screening of those mutations. Clinical and biological data were obtainable for 23 of them. Compound heterozygotes could be divided into two groups: subjects with or without iron overload. Five (22%) individuals had normal ferritin levels, whereas 18 had elevated ferritin concentrations (78%). Among those 18 patients, 7 (30% of the total) had clinical and biological criteria of genetic hemochromatosis. Eleven had iron overload without all the criteria of genetic hemochromatosis. Such a high proportion of genetic hemochromatosis is not found in heterozygotes for the C282Y mutation alone neither in our series nor in the literature. Compound heterozygotes for the C282Y and the H63D mutations may have a higher risk of iron overload or genetic hemochromatosis than single heterozygotes for the C282Y mutation. We propose a schematic theoretical representation that could explain this fact at the protein level. Further fundamental studies on the protein, and clinical follow up of compound heterozygotes could help to ascertain this hypothesis.

Keywords: iron, HFE gene, hemochromatosis, compound heterozygotes

INTRODUCTION

Two different nucleotide substitutions have been described on the DNA sequence of the HLA H (or *HFE*) gene which is believed to be responsible for hereditary hemochromatosis

(genetic hemochromatosis) (1). There is now clear evidence that the G to A transition at nucleotide 845 of the cDNA (845A) that replaces the cysteine at codon 282 by a tyrosine (mutation C282Y), is a disease-causing mutation as more than 80% genetic hemochromatosis patients are

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1079-9796/97

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Published by Academic Press
Established by Springer-Verlag, Inc. in 1975

homozygous for this mutation (1,2,3,4). In contrast, the part played by the second substitution, a C to G change at nucleotide 187 (187G or H63D), is more elusive. The heterozygous state for this mutation is highly prevalent in the general population (2,3). It has been found at a higher rate in a group of heterozygous patients for the C282Y substitution than in control individuals (1,5). It has also been described in the homozygous state in a few genetic hemochromatosis patients by different authors (1,2,3). On the other hand some authors (4) failed to find it among the patient group. Thus, this mutation is stated to be either a neutral polymorphism (4), or a mutant that could act as a worsening factor for the C282Y mutation (5, 6). In an attempt to discriminate between these two hypotheses we have studied patients who are compound heterozygotes for both mutations and have compared their clinical and biological features to those of patients who are solely heterozygous for the C282Y mutation.

PATIENTS AND METHODS

Patients

From August 1996 to April 1997, 277 blood samples were referred to our laboratory for the diagnosis of genetic hemochromatosis mutations. They were from 1) patients with a clinical picture of genetic hemochromatosis (n=99); 2) patients with biological features of iron overload and/or suspected to be affected by genetic hemochromatosis; and 3) relatives of genetic hemochromatosis patients. Blood samples from 60 control individuals belonging to the medical staff of our hospital were also tested. All of them (patients and controls) gave their written consent in accordance with French law. The criteria for diagnosis of genetic hemochromatosis were as follows: absence of any other cause of iron overload, evidence of major iron overload removed by regular phlebotomies, and in most cases, more than one affected individual in the pedigree.

Methods

DNA analysis. To investigate both substitutions we have designed a simple and reliable test based on the modification of natural restriction sites (7).

Genotypes. (Table 1) Among the referred samples we found 81 unrelated homozygotes and 36 heterozygotes for the C282Y mutation. Eighteen were homozygous for the H63D substitution while 40 were heterozygous for this substitution. A total of 29 subjects were found to be compound heterozygotes for the C282Y and H63D alleles. A single compound heterozygote was identified among the control group (1/60).

Collection of data. Clinical and biological data concerning the groups of 29 compound heterozygotes and 36 heterozygotes were collected using a questionnaire addressed to the physicians or filled in by one of us using the hospital medical file of the patient, when available.

RESULTS

Compound Heterozygotes

A total of 29 compound heterozygotes was found in a selected population of 227 subjects with disturbances of iron metabolism, with genetic hemochromatosis, or with a family history of genetic hemochromatosis. They represented 10.5% of this sample (Table 1). On the other hand, only 1 compound heterozygote was diagnosed in a control group of 60 healthy volunteers (1.7%). Thus the percentage of compound heterozygotes was 10-fold higher in the selected population than in the general population.

Data could be obtained for 23/29 compound heterozygotes from the patient group. These subjects were classified into two categories: individuals with or without abnormalities of iron parameters (Table 2). The group of individuals

with normal ferritin levels consisted of 5 subjects: 2 males and 3 females (median age 41, range 21-49). All but one were screened because of the existence of a family member affected by genetic hemochromatosis.

The second group was composed of 18 patients with high ferritin level, and no evidence of any inflammatory syndrome or malignancy. Transferrin saturation was also recorded when available (Table 2).

Eleven out of these 18 patients were referred because of abnormalities of iron metabolism (median age 59, range 32-70). All of them had ferritin levels above the normal range (median 552 µg/L, range 378-1413). Three suffered from complex pathologies including VHC related hepatitis, monoclonal gammopathy, or sideroblastic anemia.

The seven remaining patients of this second group (median age 55, range 41-72) had a

diagnosis of genetic hemochromatosis according to the pre-cited criteria. All seven had been treated by periodic phlebotomies for at least one year. Median ferritin level in the genetic hemochromatosis group was high at diagnosis (median 970 µg/l, range 436-1739).

Heterozygotes for the C282Y Mutation

Most of the subjects found to be heterozygotes for the C282Y mutation were screened in the context of family studies (26/36). Twenty five out of 36 were unrelated. Data could be obtained for only 16 unrelated individuals (Table 3). Ten of them (5 males / 5 females) had normal ferritin levels. Five (sex ratio 2/3) had an elevated serum ferritin level (median 368, range 266-1077). Only one heterozygote of our series had a diagnosis of genetic hemochromatosis and was regularly phlebotomized since 1993.

Table 1. Genotypes for all the referred subjects, patients with genetic hemochromatosis and controls used in this study. Only six genotypes are encountered, as the 845A and 187G mutations are never found on the same chromosome (2).

Genotype	C282Y+/+ H63D -/-	C282Y+/- H63D -/-	C282Y-/- H63D+/+	C282Y-/- H63D+/-	C282Y+/- H63D+/-	C282Y-/- H63D-/-
All referred subjects N=277	86 (81 unrelated)	36	18	40	29 (10,5%)	68
Unrelated genetic hemochromatosis patients N=99	81 (81.8%)	1 (1%)	4 (4.1%)	3 (3%)	7 (7.1%)	3 (3%)
Controls N=60	0 (0%)	3 (5%)	3 (5%)	15 (25%)	1 (1.7%)	38 (63.3%)

+ presence of the mutation

- absence of the mutation

Table 2. Clinical and biological data for the group of compound heterozygotes for the C282Y and H63D mutations.

No. patients n=23	Median age at diagnosis (range)	Sex Ratio M/F	Liver biopsy iron (I) > 36 µmol/g	Liver biopsy ratio (I/age) > 2.5	Ferritin ng/µl median (range)	Transferrin Saturation median (range)	Phlebotomies	Family history
group 1	n=5 41 (21-49)	2/3	0/5	0/5	109 (8-278)	40% (30-55)	0/5	4/5 (genetic hemochromatosis in first degree relative)
group 2 A	n=11 59 (32-70)	8/3	3/11	1/11	552 (378-1413)	52% (17-66)	2/11 (< 1 year)	3/11 (genetic hemochromatosis, hepatoma, cirrhosis)
group 2 B	n=7 55 (41-72)	6/1	5/7	3/7	970 (436-1739)	64 % (48-78)	7/7 (> 1 year)	5/7 (3 genetic hemochromatosis, 2 hepatoma)

Group 1, is the group of subjects with normal iron metabolism, while group 2 involves all patients with abnormalities of iron parameters. This second group is further divided into two groups: the first group (A) includes individuals with iron metabolism disturbances but who did not fulfill all the criteria of genetic hemochromatosis at the time they were investigated. All patients in the second group (B) has a diagnosis of genetic hemochromatosis.

No.: number.

Table 3. Clinical and biological data for the group of heterozygotes for the C282Y mutation.

No. patients n=16	Sex Ratio M/F	Liver biopsy iron (I) > 36 µmol/g	Liver biopsy ratio (I/age) > 2.5	Ferritin ng/µl median (range)	Transferrin saturation median (range)	Phlebotomies	Family history
group 1 n=10	5/5	-	-	56 (16-266)	nd in most of them	0/10	6/10 (genetic hemochromatosis in first degree relative)
group 2 A n=5	2/3	-	-	368 (266-1077)	nd in most of them	1/10 (< 3 months)	-
group 2 B n=1	1/0	1	1	1190	67%	1/10 (> 4 years)	-

Only unrelated subjects are presented. Group 1, is the group of subjects with normal iron metabolism, while group 2 involves all patients with abnormalities of iron parameters. This second group is further divided into two groups: the first group (A) includes individuals with iron metabolism disturbances but who did not fulfill all the criteria of genetic hemochromatosis. Only 1 patient in the second group (B) has a diagnosis of genetic hemochromatosis.

No.: number, nd: not determined.

DISCUSSION

Compound Heterozygotes Seem to Have a More Severe Phenotype than Heterozygotes for the Single C282Y Mutation

We have examined a group of 23 compound heterozygotes for the C282Y and H63D mutations of the HLA-H or HFE gene. These subjects were divided into two groups: 5 non-symptomatic individuals (22%) and 18 individuals with evidence of iron overload (78%). This second group included 7 patients with a diagnosis of genetic hemochromatosis (30% of the group of 23) and subjects with iron metabolism disturbances who did not fulfill the criteria of genetic hemochromatosis at the time they were investigated. On the other hand, a single patient from the group of heterozygotes for the C282Y mutation alone had genetic hemochromatosis.

This patient is also the only heterozygote for the C282Y mutation we found in our sample of 99 patients with genetic hemochromatosis (1%), while 7 compound heterozygotes are included in that group (7%). These frequencies are in agreement with other published series (2,3). A recent study involving 1058 genetic hemochromatosis heterozygotes (8) concluded that serum ferritin concentration was higher in genetic hemochromatosis heterozygotes than in normal subjects and increased with age. However, as the HLA-H genotype was not performed in this study, there is no way to distinguish compound heterozygotes from simple heterozygotes. In any case, these authors found that clinical complications due to iron overload were extremely rare in heterozygotes, unless the patient suffered from associated pathologies such as alcohol abuse or hepatitis.

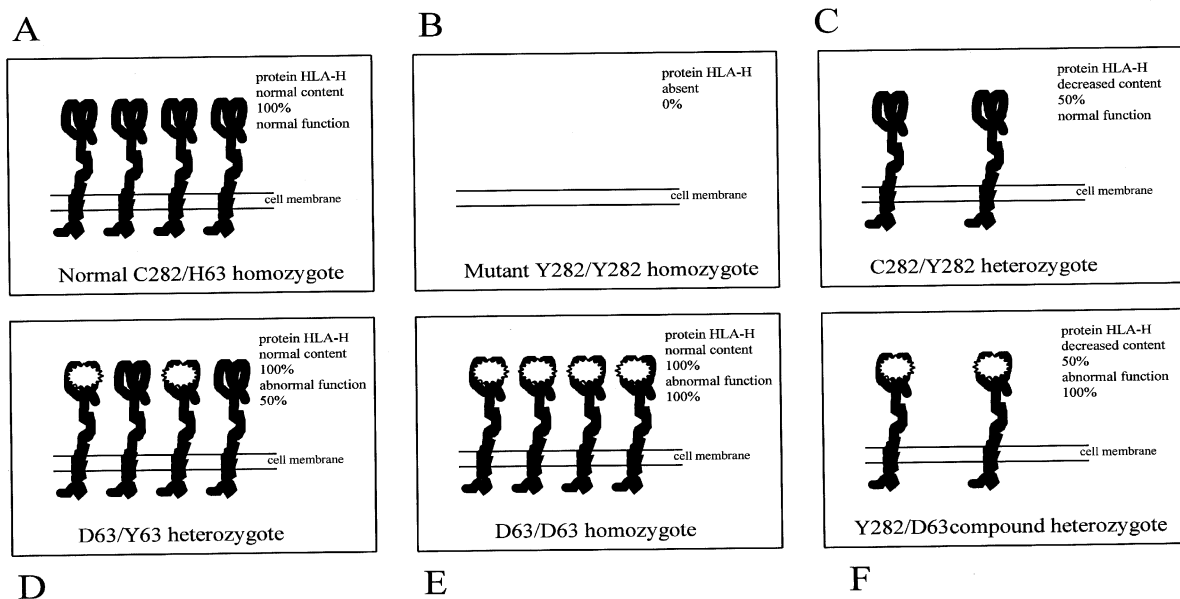


Figure 1. Schematic representation of the expression of the normal and mutated HLA H protein, hypothesized according to the model proposed by Feder et al. (1,10) and following our clinical observations. In the normal individual (A) the HLA-H protein has been demonstrated to be expressed at the cell membrane (10,11), associated with the β 2-microglobulin (β 2m) (10) [not represented in the figure]. Only six different genotypes are observed, as the C282Y (845A) and the H63D (187G) mutations are in complete linkage disequilibrium. They are never inherited together in the same chromosome (2,5). Patients homozygous for the 845A mutation (B) might have no expression of the protein at the cell surface, as the mutation is known to impair the linkage with β 2m, and thus the membrane localization. Heterozygotes (D) and homozygotes (E) for the 187G mutant could have a normal content of a qualitatively abnormal protein (respectively, 50 and 100% content). Single heterozygotes for the 845A mutation (C) could have solely a decreased content of the normal protein, whereas compound heterozygotes (F) may combine a decreased content of a functionally abnormal protein, thus manifesting a more severe phenotype.

Compound Heterozygotes Seem to Have Progressive Iron Overload in Most Cases

Seventy eight percent of the compound heterozygotes reported in this study had iron overload and thirty percent showed clinical and biological evidence of genetic hemochromatosis. Those genetic hemochromatosis patients seemed to have a late onset of the disease (median age at the diagnosis 55). In addition to the genetic hemochromatosis group, 11 compound heterozygotes had iron metabolism abnormalities but were not considered as genetic hemochromatosis. In compound heterozygotes, iron overload seemed to increase with age: median age was higher in patients with genetic hemochromatosis (55 years) or with iron overload without genetic hemochromatosis criteria (59 years), than in subjects with normal iron parameters (41 years). This age "gradient" could indicate that compound heterozygotes have a background favoring progressive iron overload. Some of them may develop true hemochromatosis. Some may not, as illustrated by the existence in that group of old patients (70 years) without clinical expression of genetic hemochromatosis. This low penetrance of the clinical expression of genetic hemochromatosis in compound heterozygotes has been recently pointed out by Beutler (5) who calculated that only about 1% of these subjects develop clinical hemochromatosis. Our series seems to indicate that clinical expression is not so rare in compound heterozygotes as 30% of the reported patients have a diagnosis of genetic hemochromatosis. Similar facts have also been underlined for homozygous individuals with the C282Y mutation: a subset of patients suffering from sporadic porphyria cutanea tarda, but not genetic hemochromatosis, are homozygous for the C282Y mutation (9). Thus the presence of the C282Y mutation, even at the homozygous state, is not fully sufficient to develop genetic hemochromatosis.

A Protein Model Could Explain the Severity of the Disease in Compound Heterozygotes

According to these findings and to the molecular structure of the protein hypothesized by Feder et al. (1) we suggest a model that might explain how the H63D variant associated with the C282Y mutant could be a worsening factor, and could favor the onset of genetic hemochromatosis (Figure 1). In this scheme, the HLA-H protein is represented as a cell membrane molecule. This was first speculated by Feder et al. (1). Recent reports have given strong evidence that the wild type HLA-H protein (10,11) and also the H63D HLA-H proteins (10) are expressed on the surface of many cells. The C282Y mutant was thought to lead to the absence of expression of the protein at the cell membrane level (1). Feder et al. (10) have demonstrated that the C282Y mutant disrupts beta 2-microglobulin interaction and cell surface expression. We can speculate that the H63D substitution could give rise to a deficient, normally expressed protein. The association of both mutations could be characterized by a lower expression (theoretically 50%) of a deficient protein, resulting in a more severe phenotype in compound heterozygotes than in heterozygotes for a single C282Y mutant. The level of expression of these defective proteins could be different among individuals, depending on genetic or environmental factors. Thus the theoretical 50% level could be either higher, resulting in the absence of clinical expression of the disease, or very low, with a clinical presentation of genetic hemochromatosis. This model could also answer another question: why do homozygotes for the H63D mutation (in our sample 18 patients, 4 of them being considered as genetic hemochromatosis) seem to have mild forms of genetic hemochromatosis, probably milder than compound heterozygotes? According to our hypothesis they may have a larger number of molecules than do compound heterozygotes even if those proteins are deficient. This situation could be compared to some "mild" mutations of the

beta-globin gene which are responsible for beta-thalassemia intermedia at the homozygous state and cause beta-thalassemia major when associated with a "severe" beta globin gene mutant.

CONCLUSION

Based on the study of a group of 23 compound heterozygotes for the C282Y and H63D mutations, we propose a schematic explanation for the role of these mutations on the HLA or HFE gene. The *in vivo* mechanisms are probably more complicated. Genetic hemochromatosis is a monogenic disease, but its expression is not straightforward, even in homozygotes with the C282Y mutation. Similar observations have been made for different monogenic diseases such as sickle cell anemia. Other genetic factors including haplotype background or association with abnormalities of other genes regulating iron metabolism can be involved. More data are needed from a fundamental or clinical point of view. Fundamental research could help to assess the validity of the model we proposed. Long term clinical and biological follow up of individuals diagnosed to be compound heterozygotes for these mutations will probably be of major practical interest.

ACKNOWLEDGMENTS

This study has been possible with the collaboration of many physicians, particularly, Drs. S Achemane, P Bonnet, S. Brun, A Dendale, JM Eiperier, T Lavabre, M Navarro, G Pageaux, G Perney, JF Rossi, and was supported by a grant from the Association Hemochromatose France.

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