

# Mutation Analysis in Hereditary Hemochromatosis

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**ABSTRACT:** The DNA of 147 patients of European origin clinically diagnosed with idiopathic hemochromatosis and 193 controls was examined for mutations of the HLA-H gene at nt 845 and nt 187. One hundred twenty-one (82.3%) of the hemochromatosis patients were homozygous and 10 (6.8%) heterozygous for the 845A (C282Y) mutation. All of the homozygous patients were also homozygous for nt 187C, and all 845A heterozygotes had at least one copy of 187C. Thus, the nt 845 and nt 187 mutations were in complete linkage disequilibrium; nt 187 was a C on all chromosomes with the 845A mutation. Eight of the 10 heterozygotes for 845A were heterozygous for 187G(H63D). The excess of heterozygotes at both nt 187 and nt 845 suggested either the presence of as yet undiscovered mutations existing in trans with 845A and in linkage disequilibrium with 187G, or that the 187G itself is a deleterious mutation, which in concert with the 845A can give rise to hemochromatosis. None of the 193 normal controls were homozygous for 845A and 29/193 (15%) were heterozygous for 845A. Although 47/193 (24.3%) of normal controls were heterozygous for the 187G mutation only two of these carried the 845A mutation. If the 187G mutation complemented the 845A mutation with high penetrance in causing hemochromatosis, then the population frequency of the two genes would require that a high proportion of patients with hemochromatosis be heterozygous for 845A and 187G. Instead, the frequency of homozygotes for the 845A mutation was much higher than that of the 845A/187G genotype. Based on our data, the penetrance of the 845A/187G genotype is only 1.5% and based on the data of Feder et al. only 0.5%. In contrast, the penetrance of the homozygous 845A/845A genotype seems to be very high. Thus, screening for this genotype should be very useful.

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## INTRODUCTION

Hereditary hemochromatosis is arguably the most common genetic disorder of Northern Europeans. Since the discovery that the disease is HLA-linked in 1976 (1), there has been an intensive effort to find the gene responsible for this disease. Recently, Feder et al.(2) described a mutation, 845A (c845A; C282Y; GenBank U60319 OMIM235200.0001) of an HLA-like gene on chromosome 6 that they designated HLA-H. This mutation was found on 85% of the chromosomes of patients with hereditary hemochromatosis and in 3.2% of those in controls of European origin who did not have the disease. A second mutation described by Feder et al. in HLA-H, 187G (c187G; H63D; GenBank U60319 OMIM 235200.0002), had an undetermined relationship to hereditary hemo-chromatosis. No other candidate mutation was identified in a 250 kb region surrounding this mutation. Moreover, the 845A mutation of HLA-H predicts a cysteine to tyrosine substitution in a region that is highly conserved in HLA-like molecules and which may preclude the appearance of HLA-H on the cell surface. Accordingly, it seems highly probable that the 845A mutation is indeed the one responsible for hemochromatosis, although the mechanism by which it produces a defect in iron absorption is obscure.

We now verify the fact that the 845A mutation is, indeed, present in a high percentage of patients with hemochromatosis and confirm that the 187G mutation is particularly common in heterozygotes for 845A who have clinical iron overload.

## MATERIALS AND METHODS

### *Patients*

DNA from 147 unrelated adult patients of European origin who had been diagnosed as having hereditary hemochromatosis are included in this study. Clinical diagnoses of hemochromatosis were based on serum iron and ferritin levels, liver biopsies, and the response to phlebotomy. As indicated below, some of these

tests had not been carried out on all of the patients. However, in each case the diagnosis of hemochromatosis appears to have been reasonably well established.

In addition DNA was obtained from 3 adult patients of Asian origin and from fibroblasts from four infants classified as having "Idiopathic Hemochromatosis, Neonatal Giant Cell Hepatitis - 231100" (NIGMS Human Genetic Mutant Cell Repository, Coriell Institute for Medical Research, 401 Haddon Ave. Camden, NJ; GM09498, GM09893, GM09811, GM09564).

DNA from 193 normal donors of European origin was also obtained.

### *Mutation Analysis*

**DNA isolation.** DNA was isolated from peripheral blood leukocytes and cultured fibroblasts using conventional methods.

**DNA amplification.** Two portions of the HLA-H gene were amplified separately using the polymerase chain reaction (PCR). The 25  $\mu$ L system contained 34 mM Tris-HCl pH 8.8, 8.3 mM ammonium sulfate, 3.4 mM MgCl<sub>2</sub>, 85  $\mu$ g/ml bovine serum albumin, 5% dimethyl sulfoxide, 0.2mM of each dNTP, 0.5 U Ampli Taq DNA polymerase (Perkin Elmer, Branchburg, NJ), 0.2 $\mu$ g genomic DNA and 75ng of each oligonucleotide primer in Table 1. The PCR was carried out for 30 cycles (94 C for 30 s, 55 C for 30 s, and 72 C for 30 s).

**Allele specific oligonucleotide hybridization (ASOH).** Three microliters of the PCR product were spotted in duplicate on nylon membranes (Oncor, Gaithersburg, MD). The membranes were soaked for 2 minutes in 1.5M NaCl/0.5M NaOH, for 2 minutes in 1.5M NaCl/ 0.5M Tris-HCl pH 8.0 and then rinsed in 0.2M Tris-HCl pH 7.5 and 2X SSC. They were then cross-linked in a UV Stratalinker (Stratagene, La Jolla, CA). Oligonucleotides matching the normal and mutant sequences (Table 1) were labeled with  $\gamma$ -<sup>32</sup>P-ATP and polynucleotide kinase (New England Bio Labs Inc., Beverly, MA) according to the manufacturers

instructions. The spotted nylon membranes were rehybridized at 42 C in heat seal bags containing 6X SSC, 5X Denhardt's solution, 0.02M sodium phosphate pH 7.0, 0.5mg/ml salmon sperm DNA and 1% SDS for 30 minutes. The prehybridization solution was removed and  $5 \times 10^6$  cpm of labeled oligonucleotide probe was added in a hybridiza-

tion solution containing 7X SSC, 0.02M sodium phosphate pH 7.0, 0.5mg/ml salmon sperm DNA and 1% SDS. The probes were annealed for two hours at 42 C and then the membranes were washed in 6X SSC and 0.1% SDS for 20 minutes at the temperatures listed in Table 1.

**Table 1.** Primers and probes used in the detection of the 187G and 845A mutations

Mutation	PCR Primers	ASOH Oligonucleotides	Wash Temperatures
187 C-G	5'ACATGGTTAAGGCCTGTTGC3' 5'GCCACATCTGGCTTCAAATT3'	5'TCTATGATCATGAGAGT3' 5'TCTATGATGATGAGAGT3'	48 C 48 C
845 G-A	5'TGGCAAGGGTAAACAGATCC3' 5'CTCAGGCACTCCTCTCAACC3'	5'ATATACGTGCCAGGTGG3' 5'ATATACGTACCAGGTGG3'	53 C 50 C

**Table 2.** Genotypes at nt 845 of adult European hemochromatosis patients and normal subjects

Genotype at nt 845	Hemochromatosis	Normal
A/A	121 (82.3%)	0 (0%)
G/A	10 (6.8%)	29 (15.0%)
G/G	16 (10.9%)	164 (85.0%)

### Nomenclature

According to a recently proposed systematic nomenclature (3) the mutations are designated by their cDNA nucleotides, beginning with 1 at the start ATG. There are four possible haplotypes with respect to the A/G polymorphism at nt 845 and the C/G polymorphism at nt 187: 187C,845G; 187C,845A; 187G,845G; and 187G,845A. Since, as shown below, complete linkage disequilibrium was found to exist between these nearby loci, the

last of these haplotypes does not exist. We therefore use the following simplified notation to designate haplotypes:

187C,845G is designated 187C

187C,845A is designated 845A

187G,845G is designated 187G

This notation simply assumes that the nucleotide at the position not given is the normal G at nt 845 or the normal C at nt 187.

## RESULTS

### *Genotypes*

The genotypes at nt 845 of the adult European patients and normal controls are summarized in Table 2. Table 3 presents the genotypes at nt 187 of the adult European hemochromatosis subjects and of the normal controls. The four infants with infantile hemochromatosis all manifested the normal 845G/845G genotype. Three were found to be 187C/187C, while one (GM09564) was 187C/187G. Three Asian patients were all found to be 187C/187C.

### *Clinical Associations*

The criteria upon which a diagnosis of hemochromatosis was based and other patient characteristics are summarized in Table 4.

### *Haplotypes*

Polymorphic markers in the area of chromosome 6 telomeric to HLA-F were determined. These data are summarized in Table 5. Haplotypes were based upon the four common markers in the region immediately centromeric to HLA-H. The hemochromatosis "ancestral

haplotype" (designated "H") represents alleles 3, 3, 4, and 5(4) for markers D6S306, D6S464, D6S1260, and D6S1588 respectively.

## DISCUSSION

Feder et al. (2) have provided strong evidence that the gene they designated HLA-H is responsible for hereditary hemochromatosis. However, the location of the gene and the fact that it is HLA-related have raised some doubts (5). HLA-H is considerably more telomeric than the markers that have shown the greatest degree of linkage disequilibrium in the hands of others (4,6-11). This may have been due to the high saturation of markers in the linkage disequilibrium analysis that Feder et al. (2) derived from an extensively sequenced region generally recalcitrant to cloning and absent from available YAC collections. Although it has been suggested that  $\beta 2$  microglobulins may be involved in some way in the etiology of hereditary hemochromatosis (12), there is no known direct relationship between any HLA-like genes and iron metabolism. Moreover, the mutation is in a domain that is highly conserved in HLA molecules, but no nonsense mutations have as yet been identified.

**Table 3.** Genotypes at nt 187 of hemochromatosis patients and normal subjects of European ancestry

Genotype at nt 845	Genotype at nt 187	Hemochromatosis	Normal
A/A	C/C	121	0
	C/G	0	0
	G/G	0	0
G/A	C/C	2	27
	C/G	8	2
	G/G	0	0
G/G	C/C	10	112
	C/G	4	45
	G/G	2	7

**Table 4.** Criteria used in the diagnosis of hemochromatosis

Hemochromatosis Genotype	Serum Iron		Serum Ferritin		Liver Biopsy		Phlebotomies			Median Age	Sex Ratio (M/F)			
	>150µg/dL	<150µg/dL	? <sup>‡</sup>	>600µg/L	<600µg/L	? <sup>‡</sup>	Done	Not done	? <sup>‡</sup>			>10	<10	? <sup>‡</sup>
845A/845A	74	5	42	66	24	31	80	17	24	86	5	30	60	70/51
845A/187C	0	0	2	2	0	0	1	0	1	0	0	2	52	2/0
845A/187G	5	1	2	5	1	2	4	2	2	4	1	3	62	8/0
187C/187C	5	3	2	10	0	0	6	4	0	7	3	0	60	6/4
187C/187G	1	0	3	3	0	1	3	0	1	2	0	2	74	3/1
187G/187G	0	1	1	2	0	0	0	2	0	1	0	1	51	2/0

**Table 5.** Chromosome 6 haplotypes of hemochromatosis patients

Hemochromatosis Genotype	H/H <sup>*</sup>	H/O <sup>†</sup>	?H <sup>‡</sup> /O	O/O	No data	Total
845A/845A	41	22	26	7	25	121
845A/187C	0	0	1	1	0	2
845A/187G	0	1	5	1	1	8
187C/187C	0	1	3	6	0	10
187C/187G	0	0	0	3	1	4
187G/187G	0	0	0	2	0	2

<sup>\*</sup>H represents the ancestral haplotype at D6S306, D6S464, D6S1260, D6S1558.

<sup>†</sup>O indicates that the ancestral haplotype is not present.

<sup>‡</sup>?H indicates that the ancestral haplotype may be present, but cannot be assured in the absence of family studies.

*The 845A Mutation in HLA-H is Strongly Associated with Hereditary Hemochromatosis*

Our data confirm that the 845A mutation of HLA-H is very strongly associated with hereditary hemochromatosis. Approximately 80% of the 147 patients that we genotyped and none of the 193 controls were homozygous for this mutation. It seems highly unlikely that the 845A mutation is a benign mutation that is in linkage disequilibrium with the true hemochromatosis gene. In a 250 Kb region surrounding this mutation, Feder et al. (2) found no candidate mutations in the approximately 15 genes they investigated other than the C-G transversion at nt 187.

*All Chromosomes That Carry the 845A Mutation Have a C at nt 187*

The mutation at nt 187 is, as would be expected by its proximity, in linkage disequilibrium with the 845A mutation: all hemochromatosis patients homozygous for the 845A mutation were found to have the 187C/187C genotype.

*There is a Great Excess of 187G Alleles Among Hemochromatosis Patients Who are Heterozygotes for 845A*

Because of the complete linkage disequilibrium of the 845A and 187C alleles, only chromosomes that are 845G should be considered "at risk" for the 187G mutation. From this perspective, table 3 shows that there are 42 hemochromatosis chromosomes at risk, and of these 16 bear the 187G allele (0.38). Among the controls 357 chromosomes are at risk and only 61 bear the 187G allele (0.17). The difference is even more striking when we consider the patients who are heterozygous for 845A. Among the 10 patients with hemochromatosis, 8 also carried the 187G allele, compared with 2 of 29 of the controls. In the study of Feder et al. 8 of 9 heterozygotes for the 845A mutation carried the 187G mutation.

*The Number of Compound 845A/187G Heterozygotes Fall Far Short of Hardy-Weinberg Predictions*

Is the less common 187G allele a mutation that contributes to the hemochromatosis phenotype? In our control population the frequency of the 845A allele was 0.075 (29/386) and that of the 187G allele 0.16 (61/386). The Hardy-Weinberg equilibrium would predict that in the general population the frequency of 845A/845A homozygotes would be 0.0056, a value fairly close to several estimates of the incidence of hemochromatosis in the European population(13-17). However, the incidence of compound heterozygotes (845A/187G) would be much higher, viz., 0.024.

*Low Penetrance of the 845A/187G Genotype May Account for the Shortfall in 845A/187G Hemochromatosis Patients*

At the calculated homozygote frequency of 0.0056, our patients would represent those drawn from a total population of 21,607. If all of the 845A/187G heterozygotes calculated to be in this population had hemochromatosis, there would have been 518 such patients in this cohort; instead there were only 8 (Table 3). From these considerations we would calculate that the penetrance of this compound heterozygous genotype is only of the order of 1.5%. Supporting these calculations is the fact that 1% of our normal control subjects had this genotype; surely there are not this many undetected hemochromatosis patients in the normal population. When the same calculations are carried out on the data reported by Feder et al. an even lower penetrance is computed, largely because the 845A gene frequency in our controls (0.075) is much higher than the frequency they report (0.032). Consequently one would calculate that their homozygotes represent a population of 144,531 and the eight 845A/187G heterozygotes they found only represents 0.5% of the expected (2).

It is also of interest that seven homozygotes for the 187G allele were found in the normal population (3.6%) and only two among the hemochromatosis patients (1.4%). While we have not had the opportunity to evaluate the iron status of the 7 patients with the 187G/187G genotype in the normal group, we would expect it to be normal.

It is likely that the combination of 845A and 187G produces a much milder disease state than the 845A/845A genotype. The fact that all of the 845A/187G patients were males is consistent with this view.

This situation is roughly analogous to some of the  $\beta$  globin chain mutations that are related to sickle cell disease. Thus, homozygosity for sickle hemoglobin produces a severe anemia, homozygosity for hemoglobin C an essentially benign state, and heterozygosity for hemoglobin S and hemoglobin C a sickling disorder which, although clinically important, is often less severe than the homozygous state for sickle cell hemoglobin.

*It is Likely that Other Hemochromatosis-Producing Mutations Exist at the HLA-H Locus*

Apart from the nt187 polymorphism Feder et al.(2) did not find any mutation on the other HLA-H allele of patients who were heterozygous for the 845A mutation. Moreover, if there were a second mutation that accounted for the disease in most of the patients heterozygous for 845A, one might expect to find linkage disequilibrium with other nearby chromosome 6 markers in this subset of patients. None was found in our study (data not shown) or in those reported by Feder et al. (2). It is possible therefore that many patients who do not have the 845A/845A genotype have hemochromatosis that is due to a mutation at a different locus.

We have pointed out previously (18) it is highly likely that other mutations exist in HLA-H. There are few if any disease-producing genes in which only one or two mutations are found.

Sequencing of the 845G allele in patients with the 845A/845G genotype will reveal whether, in point of fact, additional mutations are present. The finding of such additional mutations would help to lay rest the nagging concerns (5) that HLA-H might not actually be the long sought after hemochromatosis gene, but merely a bystander that has mutations in marked linkage disequilibrium with the real disease-producing gene.

*The Potential Utility of Population Screening*

The high proportion of patients with hereditary hemochromatosis who have the 845A/845A genotype and its virtual absence from a normal population provides us with a potentially powerful tool for population screening. Unlike serum iron and ferritin levels, that have been used previously for screening, the genotype is invariable, not being affected by factors such as liver disease and inflammation, which can modify the levels of serum iron and of ferritin both in hemochromatosis and normal subjects. As in the case of other genetic diseases, one would predict that the phenotype of the homozygotes would vary markedly. Some would probably never develop clinical disease. Yet, the treatment of hemochromatosis by phlebotomy is so simple and effective that it would appear prudent to consider a therapeutic phlebotomy program in persons discovered to have the homozygous genotype, or, at the very least, to follow their iron and ferritin levels. On the other hand patients with the 845A/187G genotype are much less likely to develop clinical disease. The data that we have developed suggested that less than 2% of the patients with this genotype become hemochromatosis patients, and this statistic must be taken into account when deciding how to deal with such patients. Assessment of the serum iron and ferritin levels of persons with this genotype every few years would seem prudent until such a time that better predictive measurements are available.

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