

# HLA-H and Associated Proteins in Patients with Hemochromatosis

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## ABSTRACT

**Background:** The 845A(C282Y) mutation in the *HLA-H* gene accounts for most cases of hereditary hemochromatosis in patients who are of European origin. Some lack this mutation, however, and it is not present in Asian patients. Thus, other mutations either in *HLA-H* or associated proteins may be present in such patients. *HLA-H* associates with  $\beta$ -2-microglobulin. Calreticulin associates with class I HLA proteins and appears to be identical with mobilferrin, a putative iron transport protein. These two proteins are therefore candidates for mutations in patients with hemochromatosis.

**Materials and Methods:** We have sequenced the coding region and parts of introns of the *HLA-H* gene, the  $\beta$ -2-microglobulin gene, and the calreticulin (mobil-

ferrin) gene of 10, 7, and 5 hemochromatosis patients, respectively, selecting those who were not homozygous for the 845A(C282Y) mutation. The number of chromosomes at risk studied were 18 for *HLA-H*, 14 for  $\beta$ -2-microglobulin, and 10 for calreticulin.

**Results:** We detected 3 new intronic polymorphisms in the *HLA-H* gene, each a point mutation. Some differences from published sequences of  $\beta$ -2-microglobulin and calreticulin were documented, but these were uniformly present in all samples.

**Conclusions:** The lack of additional mutations in the *HLA-H* gene is remarkable, and we speculate that the C282Y mutation may be a gain-of-function change.

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## INTRODUCTION

Hereditary hemochromatosis is a disorder of iron metabolism characterized by the accumulation of large amounts of iron in the parenchymal cells of the liver, cirrhosis, diabetes, heart disease, and arthropathies. The genetic linkage of this disease to *HLA-A* and *HLA-B* has been recognized for over 20 years (1). However, it was only in 1996 that the *HLA-H* gene was identified and the mutations that give rise to *HLA*-linked hemochromatosis were found (2).

Most patients with hereditary hemochromatosis are homozygous for the 845G $\rightarrow$ A (845A; C282Y) mutation of the *HLA-H* gene. In addition, some patients are compound heterozygotes for this mutation and a 187C $\rightarrow$ G (187G; H63D) mutation of the *HLA-H* gene. In most studies, however, 10% to 15% of Caucasian patients have

neither of these genotypes (2–5). Among Asian patients with hemochromatosis none have been found to have the C282Y mutation (3).

The cause of hemochromatosis in patients without the C282Y/C282Y or C282Y/H63D genotypes is unclear. It is possible that they have other mutations in *HLA-H*, although Feder et al., in their original report (2), found none. The cysteine  $\rightarrow$  tyrosine substitution at amino acid 282 is believed to impair  $\beta$ -2-microglobulin binding of *HLA-H*, and it has been found that  $\beta$ -2-microglobulin-deficient mice accumulate iron. Indeed, it has been suggested that they represent an animal model of human hemochromatosis (6,7). Another protein, calreticulin, is also of interest. This protein not only binds to *HLA* class I molecules (8) but is thought to be identical to mobilferrin, an iron transport protein (9).

Accordingly, we have examined the DNA sequences of *HLA-H*,  $\beta$ -2-microglobulin, and calreticulin in a group of hemochromatosis patients, most of whom lacked the typical hemochromatosis genotype.

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TABLE 1. Primers and Conditions for Sequencing HLA-H,  $\beta$ 2-microglobulin, and Calreticulin

Gene		PCR primers	Annealing temperature	Sequencing primers
HLA-H	Exon 1	5'-CATTGCGAAGCTACTTTCCC-3' 5'-AGTTTCGATTTTCCACCCC-3'	56°C	PCR primers
	Exon 2a	5'-acatgggtaaggcctgttc-3' 5'-GCCACATCTGGCTTGAAATT-3'	55°C	PCR primers
	Exon 2b	5'-CATGAGAGTCGCCGTGTG-3' 5'-cagctgttcctcaagatgc-3'	55°C	PCR primers
	Exon 3a	5'-tttggtgcagttaacaaggc-3' 5'-GAATCTTGTGCCTTTCCAC-3'	55°C	PCR primers
	Exon 3b	5'-GGATTGGAGAGCAGCAGAAC-3' 5'-aaactccaaccaggatcc-3'	56°C	PCR primers
	Exon 4	5'-tggcaagggtaaacagatcc-3' 5'-ctcaggcactcctcaacc-3'	58°C	PCR primers
	Exon 5	5'-gcctgaggaggtatatggc-3' 5'-gagactcccccttgttct-3'	58°C	PCR primers
	Exon 6	5'-tgggtgaatagggaaaataagg-3' 5'-ctagggatcaccggcatg-3'	55°C	PCR primers
$\beta$ 2-microglobulin	Promoter-exon 1	5'-tcctgattggctgggcacgc-3' 5'-cagagcgggagggtaggaga-3'	63°C	PCR primers
	Exon 2	5'-accctggcaatattaatgtgc-3' 5'-tacacaacttcagcagcttac-3'	59°C	PCR primers
	Exon 3	5'-ttttagacattttagtacatgg-3' 5'-ctgtaggattctttccctg-3'	58°C	PCR primers
Calreticulin cDNA	Exons 1-3	5'-CCGTCCGTCCTGACAGAGC-3' 5'-ACAGCTTCACATAGCCGCC-3'	60°C	5'-CAGAGCCGCTGCCGAGGGT-3' 5'-CCCACAGTCGATGTTCTGCT-3'
	Exons 3-5	5'-CACGCTTTTATGCTCTGTCG-3' 5'-AGTCCTCAGGCTTGGAGTC-3'	58°C	5'-TGTCGGCCAGTTTCGAGCCT-3' 5'-GTGGGATCATCGATCTTGGC-3'

Exons 5-8	5'-ACGATTGGGACTTCTGCCC-3'	58°C	5'-CCCAAGAAGATAAAGGATCCT-3'
	5'-GCCCTGTGCTCCTCGTCCCTG-3'		5'-CTCGTCTGTTTGTCTTCA-3'
Exons 8-9	5'-GGCACATTGGATCCACCCAG-3'	57°C	5'-GGAGTTTGGCAACGAGACGT-3'
	5'-TTGGCGGGCAAGCTCTG-3'		5'-TCTGCGGCAGGAGCGCTCAG-3'
Promoter	5'-ggtcctccggctcccgctgg-3'	60°C	5'-iccggcagcggctctgcagt-3'
	5'-cgcggccctttaaaccac-3'		
Exons 1-5	5'-CCGTCCGTCACCTGCAGAGC-3'	60°C	5'-CAGAGCCCGCTGCCGGAGGT-3'
	5'-ACAGCTTCACATAGCCGCC-3'		5'-CCCACAGTCGATGTTCTGCT-3'
Exons 5-7	5'-ACGATTGGGACTTCTGCCC-3'	59°C	5'-CAGCGTTTTATGCTCTGTCC-3'
	5'-ctcacCTGCCAGAGGTCC-3'		5'-GTGGGATCATCGATCTTGGC-3'
Exons 8-9	5'-tttgagtcagactcacctgg-3'	57°C	PCR primers
	5'-GGCACTTGGATCCACCCAG-3'		5'-GGAGTTTGGCAACGAGACGT-3'
Exons 8-9	5'-ggttccttctctctctg-3'	57°C	5'-TCTGCGGCAGGAGCGCTCAG-3'
	5'-TTGGCGGGCAAGCTCTG-3'		5'-gggttctctctctctctg-3'

**IVS 2 (+4) t/c**

Exon 2-AACCACAGCAAGGgta<sup>t</sup>gtggagagggggcctcaccttctgaggtgtcagagctttt

**IVS 4 (-44) t/c**

gggtgggc<sup>t</sup>gagggtggcaatcaaaggcttaactgctttttctgttttagAGCCCTCACCGT-Exon 5

**IVS 5 (-47) g/a**

caagatg<sup>g</sup>atgccttagtttgtgatgcctctttctgggtctctgtctccacagGAGGAGCCAT-Exon 6

**FIG. 1. HLA-H intron polymorphisms**

## MATERIALS AND METHODS

### Patients

Fourteen patients of European extraction and 4 patients of Asian extraction who had been diagnosed with hemochromatosis and who were not homozygous for the HLA-H C282Y mutation are included in this study. Clinical diagnoses of hemochromatosis were based on either serum iron and ferritin levels, liver biopsies, response to phlebotomy, or a combination of these. Whenever possible, information was obtained about a family history of hemochromatosis, but such a history was obtained unequivocally for only 1 patient from whom all genes were sequenced.

### Polymerase Chain Reaction (PCR) Amplification and Sequencing

Genomic DNA was extracted from peripheral blood leukocytes using conventional methods. Total RNA was isolated from peripheral blood leukocytes with Trizol (GIBCO/BRL, Gaithersburg, MD).

The exons of HLA-H were amplified from genomic DNA using polymerase chain reaction (PCR) with primers based on the sequence of Ruddy et al. (10) as deposited in GenBank as U91328 (Table 1).  $\beta$ -2 microglobulin exons and promoter were amplified from genomic DNA by PCR using primers based on sequences M17986 and M17987 in GenBank (Table 1). When mRNA was available, calreticulin was amplified by PCR from first-strand cDNA made using an RT-PCR kit (Stratagene, La Jolla, CA). Promoter and cDNA sequences were obtained from GenBank (V59053, S78537, M84739) and appropriate primers for amplification of the coding region and promoter were designed (Table 1). In some cases, calreticulin was amplified from genomic DNA with cDNA primers and with intronic primers from sequence data obtained using cDNA primers (Table 1).

When PCR was performed on genomic DNA,

the amplification system contained 33.5 mM Tris-HCl, pH 8.3, 8.3 mM  $(\text{NH}_4)_2\text{SO}_4$ , 85  $\mu\text{g}/\text{ml}$  BSA, 3.35 mM  $\text{MgCl}_2$ , 5% DMSO, 0.2 mM dNTPs, 250 ng of each oligonucleotide primer, and 0.5–1  $\mu\text{g}$  of genomic DNA in 100  $\mu\text{l}$ . After denaturation at 98°C for 5 min, DNA was amplified for 30 cycles at 94°C for 30 sec, 54–60°C for 30 sec, and 72°C for 30–90 sec.

PCR products were purified on QIAquick PCR purification columns (Qiagen, Chatsworth, CA) and sequenced using either nested or PCR primers on an automated sequencer (ABI, Foster City, CA).

## RESULTS

### HLA-H

The entire coding region of the *HLA-H* gene of 10 patients was sequenced. Included were 8 patients with the normal 845G/845G genotype and 2 patients who were heterozygous for the 845A (C282Y) mutation, both of whom were compound heterozygotes for 187G (H63D). All of the coding region except for a portion of exon 3 was sequenced in an additional patient with the normal 845G/845G genotype.

No additional mutations were found in the coding region of HLA-H. However, three polymorphic sites were detected in introns 2, 4, and 5. The relevant sequences are shown in Fig. 1. In studying a large number of normal subjects and patients with hemochromatosis, 6 of the 8 possible haplotypes created by these diallelic sites have been identified (data not shown).

### $\beta$ -2-Microglobulin

The entire coding region of the  $\beta$ -2-microglobulin gene of 7 hemochromatosis patients was sequenced. Included were 6 patients with the normal 845G/845G genotype and 1 patient who was a compound heterozygote for the 845A (C282Y) and 187G (H63D) mutations. There were some

differences between the sequences that we obtained and those recorded in GenBank under accession numbers M17986 and M17987. In the GenBank M17986 sequence, nucleotides 837–838 in the signal peptide are given as gg and nt 942 was given as a c. Only a single g was present at nt 837 and there was a t at nt 942 in all of our samples. In exon 2 of sequence M17987, nt 200 is given as an a; we found a c in all samples. However, GenBank sequence J00105 agrees with the sequence that we observed. In the 3' untranslated region, nt 1134 is given as a single g. We found two g's at this location.

Apart from these differences, which were found in all samples, the  $\beta$ -2 microglobulin sequence was identical to the sequences deposited in GenBank. Accordingly, it appears that there were no mutations that could account for iron storage disease in these samples.

### Calreticulin (mobilferrin)

The entire coding region and the promoter of the calreticulin gene of 5 patients was sequenced. Included were 3 patients with the normal 845G/845G genotype and 2 patients who were heterozygous for the 845A (C282Y) mutation, one of whom was a compound heterozygote for 187G (H63D). There were three differences in the sequences we obtained from the reference sequence GenBank X59053. We found that nt 165–169 were cccgc instead of gggcg, that a t at nt 201 was deleted and that two g's were found at nt 368 rather than the one given in the GenBank file. However, two other GenBank sequences, X85727 and AD000092, agreed with the sequence that we found. Moreover, we sequenced the promoter region from an additional 8 hemochromatosis patients with the 845G/845G genotype and from 3 normal subjects, finding the same sequence. Therefore, it is apparent that no mutations that could account for hemochromatosis were found in any of the samples examined.

## DISCUSSION

The discovery of the *HLA-H* gene on chromosome 6 is a major advance in the understanding of hemochromatosis. The C282Y mutation accounts for over 80% of patients with hereditary hemochromatosis with European ancestry. However, more than 10% of European patients with hemochromatosis and most Asian patients with

this disease do not have this mutation. It would be a reasonable expectation that some or all of these patients would have other mutations of this gene. In other autosomal recessive diseases in which one or more predominant mutations representing a founder effect are present, many other less common mutations producing the same disease have been detected (11–14). However, Feder et al. (2) found only the C282Y and H63D mutations to be associated with hereditary hemochromatosis; they were unable to find any other mutations in the 30 patients that they studied who were not homozygous for the C282Y. These were our finding, too, in the 10 patients with 18 chromosomes at risk that we studied. We are surprised at this finding, but with total lack of information regarding the mechanism by which the homozygous C282Y substitution causes increased iron absorption and of how the heterozygote for this mutation is complemented by the H63D substitution, one can only speculate about the lack of other mutations in *HLA-H* in hemochromatosis patients. Possibly, the C282Y mutation produces a gain-of-function rather than a loss-of-function. If this were the case, other mutations that prevented synthesis of *HLA-H* might not complement this mutation or cause a similar phenotype. The sickle cell mutation can be regarded as an example of such a gain-of-function mutation. Of the hundreds of mutations known to exist in the hemoglobin  $\beta$ -chain gene, it is only the glu $\rightarrow$ val substitution in codon 6 that supports sickling. The putative, partially dominant nature of the C282Y mutation (15,16) fits a gain-of-function model, but data concerning the phenotype of "heterozygotes" will need to be re-evaluated to separate compound heterozygotes for the H63D mutation from simple heterozygotes.

Since *HLA* class I molecules associate with other proteins during transport to the membrane, it seemed possible that mutations of such molecules might create phenocopies of the *HLA-H* mutations. The most attractive candidates for such mutations are  $\beta$ -2-microglobulin and calreticulin.  $\beta$ -2-microglobulin seems to be an outstanding candidate because it is believed to associate with *HLA-H* (2) and because targeted disruption of the murine gene is associated with iron storage (6). Calreticulin is known to associate with many proteins, including *HLA* class I molecules (8), but our particular interest in this molecule is based on the demonstration that the iron transport protein mobilferrin seems to be identical with calreticulin (9). A number of dif-

ferences were found in the sequences of these genes from those deposited in GenBank. However, these were found in the homozygous form in all samples examined. Only a few of the differences were in coding regions, and in these we found other GenBank sequences that were identical to ours. Thus, we concluded that there were no significant mutations in either of these genes in any of the hemochromatosis patients studied.

In sequencing the *HLA-H* gene we found three new polymorphic sites. All of these are intronic and are unlikely by their nature and location to cause abnormalities in splicing. We have detected 6 haplotypes of HLA-H. If the 845A and 187G hemochromatosis mutations are included, 8 haplotypes of the HLA-H gene can now be differentiated. Because these are diallelic markers, they may prove to be useful in genetic studies of this portion of chromosome 6.

## ACKNOWLEDGMENTS

This study is manuscript 10764-MEM from The Scripps Research Institute and was supported by National Institutes of Health grants HL25552 and RR00833, and the Stein Endowment Fund. We are grateful to D. A. Ruddy and colleagues at Mercator Genetics Corp. for making available intron sequences of HLA-H prior to publication.

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Communicated by E. Beutler. Accepted April 21, 1997.