

Mutation Analysis of the HLA-H Gene in Italian Hemochromatosis Patients

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Summary

Hemochromatosis (HH) is an inborn error of iron metabolism, frequent among Caucasians, characterized by progressive iron loading that, if untreated, causes high morbidity and death. HLA-H, a putative HH gene, has recently been isolated. The large majority of patients so far studied are homozygous for a single mutation, which results in a cysteine-to-tyrosine substitution at amino acid 282 of the protein. A second, less frequent, variant, His63Asp, has an undefined role in the pathogenesis of the disease. Here we report that the Cys282Tyr change accounts for 69% of HH chromosomes in a series of 75 unrelated Italian patients who fulfilled well-defined criteria for HH diagnosis. Sixty-four percent of patients were Cys282Tyr homozygous, 10% were heterozygous, and 21% carried the normal allele. The same mutation was rare in normal controls. The His63Asp variant was less frequent but had a similar frequency among affected and normal chromosomes. Subjects without two copies of the Cys282Tyr change were both isolated patients and individuals from families with a 6p-linked disease. Mutation analysis of the HLA-H gene, carried out by RNA-SSCP in the latter patients, did not reveal any significant nucleotide abnormality in coding sequences and intron-exon boundaries. The absence of mutations in HLA-H gene was confirmed in three cases by direct sequencing. Major deletions or rearrangements of the gene were excluded by Southern blotting. The existence of patients with clinical and histological features of HH, but without mutations in HLA-H gene, suggests that in Italy the disease is more heterogeneous than reported in northern Europe.

Introduction

Hemochromatosis (HH) is an autosomal recessive disorder of iron metabolism with a prevalence of 2–5/1,000 among Caucasians (Edwards et al. 1988). The disease is characterized by abnormal intestinal iron absorption and progressive increase of total body iron, which results in midlife in clinical complications including cirrhosis, cardiopathy, diabetes, endocrine dysfunctions, arthropathy, and susceptibility to liver cancer. Since the disease complications can be effectively prevented by regular phlebotomies, early diagnosis is most important to provide a normal life expectancy to the affected subjects. It has been recently shown that an HLA class I-like gene, designated "HLA-H," located 4 Mb telomeric to HLA-A, is mutated in the large majority of HH chromosomes (Feder et al. 1996). The most prevalent mutation changes the invariant 282 cysteine residue to tyrosine, a substitution predicted to disrupt a putative β_2 microglobulin-binding site in the protein. Its relevance for the disease is inferred by the model of β_2 microglobulin-deficient mice, which develop a condition of iron overload similar to HH (Rothenberg and Volland 1996). A second mutation changes histidine at position 63 to aspartic acid, though its role in the disease is controversial. Extensive studies of patients of mixed European ancestry disclosed an incidence of 83% for the Cys282Tyr mutation among patients (Beutler et al. 1996; Feder et al. 1996). Analyses of patients of northern European origin showed an even higher frequency, which reaches >90% in Brittany (Jouanoulle et al. 1996) and 100% in Australia (Jazwinska et al. 1996).

The present paper shows that the Cys282Tyr substitution in Italy accounts for 69% of HH and 1% of normal chromosomes. In patients who do not have this mutation, extensive screening of HLA-H coding sequences and flanking exon-intron junctions by means of RNA-SSCP, direct sequencing, and Southern blotting did not reveal additional mutations. The absence of mutations in the HLA-H gene in a consistent proportion of HH alleles supports the idea that HH is a genetically heterogeneous disorder.

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Patients, Material, and Methods

Patients and Controls

Seventy-five unrelated patients of Italian origin were studied. Forty-two were from well-typed HH families, most with two or more affected siblings, and 33 were isolated patients. Criteria for HH diagnosis were based on (a) absence of known causes of secondary iron overload, (b) transferrin saturation >50% and increased serum ferritin value >2 SD above the appropriate mean corrected for sex and age (Adam et al. 1995), (c) hepatic iron overload of III or IV grade according to Scheuer et al. (1962), with a homogeneous hepatocellular deposition of hemosiderin granules showing the typical gradient from portal tract to centrolobular zone (Deugnier et al. 1992), and (d) hepatic iron index >2 (Powell et al. 1994) and or a total iron removed by weekly phlebotomies >5 g in men and >3 g in females (Piperno et al. 1996).

Two series of controls were examined. The first was represented by 36 normal chromosomes obtained from obligate heterozygotes of HH families, by subtracting the affected haplotype, as reported by Camaschella et al. (1996). This selection avoids the risk of including HH alleles in random controls from the general population. The second series of controls included 50 blood donors, at their first blood donation, with normal iron parameters.

Molecular Studies

DNA was obtained by peripheral blood buffy coats, following phenol-chloroform extraction. Simple PCR-based assays were developed to detect Cys282Tyr and His63Asp mutations in the HLA-H gene on genomic DNA. Primers used to amplify the fragments encompassing the two mutations were synthesized (Feder et al. 1996). PCR was performed using automated Thermal Cycler (Perkin Elmer) in 25 μ l final volume by use of 12.5 pmol of each primer and 0.5 U of *Taq* DNA Polymerase, for a total of 30 cycles.

Aliquots of the PCR products were digested with *RsaI* and *MboI* restriction enzymes (New England Biolabs), in order to identify the Cys282Tyr and His63Asp variant, respectively. Digestion conditions were according to manufacturer's protocols. After digestion, fragments were electrophoresed on 2% agarose gels.

RNA-SSCP

Six sets of specific primers were designed after the definition of HLA-H genomic structure (authors' unpublished data), in order to amplify the HLA-H exons and flanking exon-intron boundaries (fig. 1) (Primer sequences are available on request from P.G.). One of each primer set incorporated the T7 phage promoter

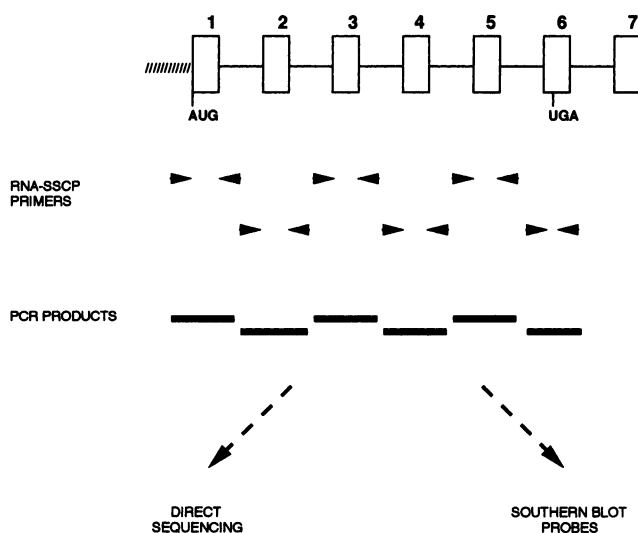


Figure 1 Scheme of the methodological approach used to screen for mutations the HLA-H gene. Exons are indicated as white boxes. The 5' flanking region is indicated by a dashed area. Primers used for RNA-SSCP analysis are indicated by arrows under the scheme of the gene structure. Amplified fragments from a normal control were used as probes for Southern blotting, and the same fragments amplified from three patients were directly sequenced.

sequence as described by Saarkar et al. (1992). Fragment size ranged from 219 to 410 bp. PCR was performed on 500 ng genomic DNA from 18 patients and 10 normal controls according to standard protocols. Transcription was carried out as described by Bisceglia et al. (1994). An aliquot of 4.5 μ l of the transcription reaction was then loaded onto a 6.5% nondenaturing polyacrylamide gel. Electrophoresis was performed at room temperature at a constant 30 w for 12 h. After electrophoresis, the gels were dried and subjected to autoradiography for 12 h.

Direct Sequencing

Direct sequencing was performed on HLA-H genomic fragments amplified by PCR (fig. 1) in three cases, by use of a Sequenase 2.0 DNA sequencing kit (USB) and an automated sequencer (Applied Biosystem 373A).

Southern Blotting

Southern blotting was performed by standard methods. Restriction-enzyme digestion (*EcoRI*, *HindIII*) was according to the manufacturer's recommendations. In the absence of a cDNA probe, filters were hybridized to a radioactive probe consisting of a mixture of amplified fragments corresponding to HLA-H exons of a normal subject (fig. 1).

Haplotype Definition

HLA typing and alleles at D6S265 and D6S105 in the families were defined as reported by Camaschella et al.

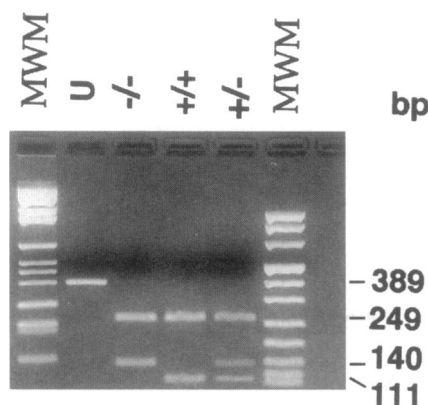


Figure 2 Identification of the Cys282Tyr mutation by *RsaI* digestion of PCR products. Band sizes (in bp) are shown on the right. U = undigested fragment; -/- = normal; +/+ = Cys282Tyr homozygote; +/- = Cys282Tyr heterozygote; MWM = molecular weight marker.

(1996). Primers sequence for D6S1260 were from Stone et al. (1994). Sequence information for D6S1621 were obtained from Whitehead Institute-MIT Center for Genome Research (<http://www-genome.wi.mit.edu/>). Microsatellites were typed as described by Camaschella et al. (1996). Haplotypes were constructed manually in 42 cases, on the basis of family segregation of microsatellite markers studied, when HH phase was unequivocally determined.

Results

Clinical and Histological Findings

Transferrin saturation was >55% in all the patients (range 56%–100%), serum ferritin ranged between 600 and 10,990 $\mu\text{g/liter}$, and liver siderosis was grade III or IV in all patients. Liver iron concentration was available in 56 patients and the hepatic iron index was >2 in all cases. All patients had completed a iron-depletion program through weekly phlebotomies. Quantitation of iron removed ranged between 4 g and 37 g.

Analysis of HLA-H Known Mutations

Amplified genomic fragments encompassing the mutation site were obtained using oligonucleotide primers as described by Feder et al. (1996). When PCR products of 389 bp encompassing codon 282 are digested with *RsaI*, fragments of 249 bp and 140 bp are obtained in normal controls. The G→A mutation at nt 845, which results in the Cys282Tyr change, introduces a new *RsaI* cleavage site, giving origin to a 111- (and 29- [not visible in figure]), instead of 140-bp, fragments (fig. 2). The His63Asp substitution was identified by an analogous approach, exploiting the C→G change at nt 187, which

abolishes an *MboI* restriction site. *MboI* cleaves the 208-bp PCR product in two fragments of 138 bp and 70 bp in the presence of the wild-type, but not the mutant, allele.

The results of the analysis of the two HLA-H mutations in patients and controls are reported in table 1. The Cys282Tyr mutation was found in 69% of the HH chromosomes and in 1% of random normal chromosomes. None of the normal chromosomes selected from HH families had this mutation. Among patients, 64% were homozygous and ~10% were heterozygous for the Cys282Tyr mutation. Twenty-eight (33%) of 84 typed chromosomes carried the ancestral haplotype associated with serotype HLA-A3 and defined by D6S265 allele-1 and D6S105 allele-8 (Jazwinska et al. 1995), D6S1260 allele-4, and, in most cases, D6S1621 allele-5. All the ancestral patient chromosomes carried a tyrosine codon at position 282, but the same mutation was observed also in nonancestral chromosomes. The His63Asp variant was observed in a similar proportion of affected and normal chromosomes. A single homozygote for this allele was present among patients, whereas five subjects were compound heterozygous for Cys282Tyr and His63Asp. Sixteen (21%) patients were homozygous for the wild-type allele. No common haplotype was observed in cases negative for Cys282Tyr mutation.

Patients without two copies of the Cys282Tyr mutation were either isolated cases or patients from families (nine cases). In four of these families with probands homozygous for the wild-type allele, the disease appeared to segregate with 6p marker. However, there was only a single affected subject and multiple unaffected

Table 1

HLA-H Genotypes Resulting from the Analysis of the Known Mutations in the Series Studied

GENOTYPE		PATIENT		RANDOM CONTROLS		FAMILY-BASED CONTROLS
C282Y	H63D	No.	%	No.	%	
++	--	48	64	0	0	
+-	--	2	2.7	1	2	
+-	++	5	7	0	0	
--	+-	3	4	10	20	
--	++	1	1.3	0	...	
...	...	16	21	39	78	
Total		75		50		
Allele Frequency (%)						
C282Y		103/150 (69)		1/100 (1)		0/36 (0)
H63D		10/150 (7)		10/100 (10)		3/36 (8)

siblings. In three multiplex families, compound heterozygosity for Cys282Tyr and His63Asp clearly segregated with the disease, suggesting a causality for this association. The last two families had two pairs of affected siblings each, with identical chromosome 6 haplotypes, who carried only His63Asp at the heterozygous state.

Screening HLA-H Gene for Mutations

The absence of two copies of the major mutation in HH families that appeared to be 6p linked suggested the presence of additional mutations in HLA-H gene. Therefore, exploiting the availability of the HLA-H genomic structure (authors' unpublished results), we amplified and screened by RNA SSCP HLA-H gene exons and exon-intron junctions from genomic DNA of 18 patients who did not carry two copies of Cys282Tyr substitution. No mobility shift was observed in the relevant sequences of the gene. The absence of mutations was confirmed in three cases by direct sequencing. No major deletion/insertion was observed hybridizing HLA-H amplified exons on *EcoRI* and *HindIII* digested DNA of 10 of these patients (data not shown).

Discussion

The Cys282Tyr mutation is highly prevalent among Caucasian patients with HH. Homozygotes for this mutation range from 83% in Northern American studies (Feder et al. 1996; Beutler et al. 1996) to >90% among Bretons (Jouanolle et al. 1996) and $\leq 100\%$ among patients of Irish-Scottish origin in Australia (Jazwinska et al. 1996). In our series, Cys282Tyr was present in 69% of HH chromosomes and had a very low frequency (1%) among normal chromosomes. The latter value is significantly lower than expected on the basis of the estimated frequency of the disease (Velati et al. 1990). This observation, associated with the finding that only 64% of patients are Cys282Tyr homozygous, suggests the possibility that HH in Italy is caused by additional molecular defects. A consistent proportion of patients in a series from southern France (Borot et al., in press) lacks the major HLA-H mutation, suggesting the possibility that HH in southern Europe is more heterogeneous than reported in northern Europe, as described in other common genetic disorders (Estivill et al. 1988; Dianzani et al. 1994).

It is remarkable that the Cys282Tyr substitution was absent in 31% of HH chromosomes. They are not accounted for by His63Asp variant, which is present in a minority of cases. Also, several lines of evidence indicate that this variant is a polymorphic change (Feder et al. 1996). First, it attains a similar frequency in patients and controls and occurs on chromosomes unrelated to

the ancestral one. Second, homozygotes for this mutation are extremely rare among patients both in our and in other series (Beutler et al. 1996; Feder et al. 1996; Jouanolle et al. 1996). Third, compound heterozygotes for Cys282Tyr and His63Arg have been observed in unaffected subjects (Jazwinska et al. 1996). Whether the interaction of the His63Asp polymorphism with the major mutation can produce a HH phenotype is controversial. On the basis of our data, which show five compound heterozygotes for the two variants among patients and none among controls, as well as a segregation of this association with the disease in three multiplex families, we cannot exclude this possibility. The event that His63Asp is linked to another causal mutation in HLA-H seems unlikely, in light of the results of HLA-H gene sequencing in the Feder series and in our cases.

Patients who did not carry two copies of the Cys282Tyr substitution represented a significant proportion of our HH patients. Since the possibility has been raised that HH misdiagnosis might explain negative cases (Jazwinska et al. 1996), clinical data and liver histology were reevaluated in all our "negative" cases, and the diagnosis was consistently confirmed. In view of the severity of iron overload (Piperno et al. 1996), the phenotypic expression in these cases was variable, from mild to severe. The absence of the putative causal mutations in affected siblings from families where the disease appeared to segregate with markers of 6p suggested the possibility of other mutations in HLA-H gene. However, no mutations were detected by RNA-SSCP in HLA-H genomic sequences. This technique is accurate and sensitive and has been used extensively to screen for mutations (Saarkar et al. 1992; Bisceglia et al. 1994). Nevertheless, to avoid the risk that some mutations might have escaped identification because of the resolution of the technique, three cases were subjected to direct sequencing, which confirmed the absence of nucleotide changes. In addition, Southern blotting results in a subset of these patients excluded the presence of large deletions, insertions, or rearrangements. In conclusion, extensive analysis of HLA-H gene in patients without two copies of Cys282Tyr substitution did not reveal new mutations within the relevant coding sequences of the gene. These results are in agreement with those reported by Feder et al. (1996), who did not find additional mutations in their negative cases. Our analysis discloses that even polymorphisms are rare in this gene, whose sequence is highly conserved.

Several possibilities remain to be explored. In cases with a 6p-linked disorder, mutations might occur in unexplored regions of HLA-H as portion of introns, RNA untranslated regions, or regulatory elements. Alternatively, the possibility of mutations outside the HLA-H

gene, but at a tightly linked locus, should be considered. In isolated patients, we cannot exclude the existence of a locus on a different chromosome as proposed by Feder et al. (1996), although, on the basis of extensive family studies, locus heterogeneity is considered unlikely in this disorder (Powell et al. 1988). We are at present exploring all these possibilities by completing the transcriptional map of the 6p region surrounding the 250-kb interval, which has been fully sequenced (Feder et al. 1996), and extending family studies to “negative” patients.

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