Spectrum of hemojuvelin gene mutations in 1q-linked juvenile hemochromatosis

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Juvenile or type 2 hemochromatosis (JH) is transmitted as a recessive trait that leads to severe iron overload and organ damage typically before age 30 years. Linkage to a locus on chromosome 1q has been found in most patients with JH. The recently identified causal gene encodes hemojuvelin, a protein with a proposed crucial role in iron metabolism. A second, rare type of JH, with clinical expression identical to the 1q-linked form, is due to inactivation of hepcidin, the key regulator of iron homeostasis. Here we report the spectrum of mutations of the hemojuvelin gene *(HJV)* in 34 patients who did not show hepcidin mutations. This represents the largest cohort of patients with JH collected worldwide. We identified 17 different (16 novel) mutations of *HJV*, both at the homozygous and at the compound heterozygous state. Mutations either generate premature termination codons or were missense substitutions, affecting highly conserved residues, relevant to the protein structure and/or function. (Blood. 2004;103:4317-4321)

Introduction

Hereditary hemochromatosis (HH) is a recessive disorder as a result of inappropriately high intestinal iron uptake that leads to progressive iron overload. The disorder is caused by mutations in genes that are hypothesized to play an essential role in the regulation of iron homeostasis.[1] HFE or type 1 hemochromatosis, the most important disease in terms of world frequency, is a late-onset, low-penetrance disorder that affects predominantly men[2-6]; all these features are consistent with a mild effect of HFE protein on inhibition of iron absorption. Type 2 or juvenile hemochromatosis (JH) is a rare form of iron overload that affects young patients of both sexes and leads to severe clinical complications typically before age 30 years.[7] Compared with HFE-related[8] and transferrin receptor 2 (TFR2)–related or type 3 hemochromatosis,[9] the clinical course of JH is rapid and severe, indicating that the JH protein is an important regulator of iron absorption.[10]

JH is genetically heterogeneous because it is associated with 2 distinct chromosomal loci. Most patients have a disorder linked to 1q.[11-13] The 1q locus was mapped in 1999 in families of Italian descent,[11] but the cognate gene remained elusive until recently.[14] Consistent with the decisive role of the JH protein(s) on iron homeostasis, a rare subset of patients with JH in which the causal gene maps to chromosome 19 has been found to harbor mutations in hepcidin.[15,16] Hepcidin encodes the key regulator of iron absorption in mice,[17-19] and the finding that hepcidin is mutated in JH suggests that the product of the 1q locus may participate in the action of hepcidin and may, in fact, represent its cellular receptor.[20]

The JH gene that maps to 1q was recently identified through an elegant positional cloning approach.[14] Mutations were reported in a previously anonymous 4 exon transcript in patients of different ethnic backgrounds. This gene was named *HFE2* and the encoded protein hemojuvelin.[14] The nomenclature of the different hemochromatosis types is controversial. We used the term "*HJV*" (Online Mendelian Inheritance in Man [OMIM] *608374) instead of *HFE2* to indicate the hemojuvelin gene. We think that the term *HFE2* should be avoided, because in the genetic nomenclature it refers also to the juvenile hemochromatosis disease that is now recognized as heterogeneous. The predicted protein, hemojuvelin, is composed of 426 amino acids and shows homology to a repulsive guidance molecule (RGM). These molecules contain several functional motifs, including a putative short transmembrane (TM) domain, a RGD motif, and a von Willebrand–like domain.[14] One frameshift, one nonsense, and 4 missense mutations in highly conserved residues proved the causality of the gene in juvenile hemochromatosis.[14] However, the role that hemojuvelin plays in the regulation of iron homeostasis is uncertain, and its putative functional relationship with hepcidin remains speculative.

To gain further insights into the structure-function relationship of hemojuvelin, we screened for mutations of the *HJV* gene coding sequences in a large series of patients with hepcidin-unrelated JH.[15]

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Submitted January 20, 2004; accepted February 11, 2004. Prepublished online as *Blood* First Edition Paper, February 24, 2004; DOI 10.1182/blood- 2004-01-0192.

Supported partially by Telethon ONLUS Foundation (grant GP00255Y01 to C.C. and grant GTF03012 to S.V.), European Union (EU) (508644 to S.V.), and the Italian Ministry of Instruction and University (FIRB and PRIN to C.C. and P.G.).

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Table 1. Sequences of the primers used for screening of HJV mutations

Primer	Sequence	PCR product length, bp
Exon 1 F	5′ CCGTCAACTCAGTAGCCA 3′	336
Exon 1 R	5′ CATTTGGACGAGAGACATC 3′	
Exon 2 F	5′ CCCCAAATTCCAGTCTGTT 3′	380
Exon 2 R	5′ CTCATTCAGGCTCACATGC 3′	
Exon 3 F	5′ CTCCGATAGAGCAGAGGTC 3′	709
Exon 3 R	5′ GGAGCATTGCTGTTGAATAG 3′	
Exon 4a F	5′ ACTATGTATGAGGTCTGATTG 3′	840
Exon 4a R	5′ CTGCAGCCTCATCTGACTC 3′	
Exon 4b F	5′ CAGCTGAACAGGACCTGC3′	1043
Exon 4b R	5′ CACATTGCATTTACTCCATTTC 3′	

The results of this study expand the spectrum of mutations that affect the hemojuvelin gene and provide insights into the residues which have an essential role in the protein.

Patients and methods

Patients

The study includes 34 patients belonging to 29 families that represent the largest series of patients with JH worldwide collected. Thirteen families were Italian, 1 was Albanian, 1 was French, 1 was from the United Kingdom, and 1 was from Australia. Clinical findings were previously reported for most of these patients,[10] and mutations in hepcidin were excluded.[15] Seventeen French-Canadian affected individuals were from 12 families of the isolated region of Saguenay-Lac-Saint Jean (Quebec)[21] and were previously shown to have a disorder in linkage with the 1q locus.[13] All patients included in the study had a clinical diagnosis of JH according to the proposed criteria.[7]

Normal controls were healthy subjects from the laboratory staff or blood donors with normal iron parameters.

Informed consent was obtained for molecular studies according to the guidelines of the different institutions.

Molecular studies

PCR and mutation detection. DNA was prepared from peripheral blood, according to standard protocols. Primers were designed to amplify coding sequences, exon-intron boundaries, and 5′ and 3′ untranslated region of the hemojuvelin gene. Sequences were obtained at http://www.ncbi.nlm.nih.gov/entrez/viewer.fcgi?val = NT_004 434.16&db = Nucleotide&dopt = GenBank and are reported in Table 1.

Polymerase chain reactions (PCRs) were carried out in a volume of 25 μL with 100 ng template DNA, 2.5 μL 10 × PCR buffer containing $MgCl_2$ (15 mM), 2 μL dNTP (deoxynucleotide triphosphate; 2.5 mM), 0.5 μL each primer (20 pM/μL), and 0.3 μL AmpliTaq Gold (Perkin-Elmer, Shelton, CT). PCRs were performed in an automatic Thermal Cycler (Perkin-Elmer 9700) under the following conditions: 95°C for 10 minutes, 30 cycles of 94°C for 30 seconds, melting temperature (Tm) for 30 seconds, 72°C for 30 seconds, and a final step of 72°C for 10 minutes.

For direct sequencing, PCR products were run on 1% agarose gel, purified using QIAquick PCR purification kit (QIAGEN, Valencia, CA), and sequenced by using Big Dye Terminator (version 3.1) cycle sequencing kit (Applied Biosystem, Foster City, CA). After purification from unincorporated dye with Autoseq G-50 columns, sequencing products were electrophoresed in an automatic sequencer (ABI Prism 3100 Genetic Analyzer; Applied Biosystem), according to the manufacturer's protocols.

Restriction digestions were carried out on 20 μL PCR products by using 10 U endonuclease (New England Biolabs, Beverly, MA) in a final volume of 30 μL for 2 hours.

DHPLC. For the screening of mutated alleles in a large series of normal control subjects, all amplicons underwent denaturing high-performance liquid chromatography (DHPLC). DHPLC was performed on a WAVE Nucleic Acid Fragment Analysis System HSM (Transgenomic, Crewe, United Kingdom).

To enhance heteroduplex formation, the untreated PCR product mixed in a 1:1 ratio with a PCR product from a wild-type control was denatured at 95°C for 5 minutes followed by gradual reannealing at 25°C over 30 minutes. Samples were initially analyzed at the Tm determined by using the Wavemaker software (Transgenomic). Eluted DNA fragments were detected by an UV-C detector (Transgenomic). Tm for all primer pairs, as well as specific protocols for the DHPLC procedure, is available on request. PCR products showing an abnormal chromatographic profile on DHPLC analysis were sequenced directly. Approval was obtained from the Dipartimento di Scienze Cliniche e Biologiche of the University of Torino institutional review board for these studies.

Results

With the study of a large series of families with JH we have identified 17 mutations (16 of which are novel) in the *HJV* gene. The whole list of mutations detected and their effect on the protein

Table 2. List of mutations found in HJV and their occurrence on different alleles

Nucleotide change	Exon	Residue	Family origin	Alleles, no.	Homozygotes, no.	Restriction enzyme
220delG	3	V74fsX113	English	1	0	NA
253T > C	3	S85P	Italian	2	1	*Bsr*BI
295G > A	3	G99R	Albanian	1	0	*Sac*II
302T > C	3	L101P	Albanian	1	0	*Mnl*I
391-403del	3	R131fsX245	Italian	2	0	NA
445delG	3	D149fsX245	Italian	6	3	*Mae*III
503C > A	3	A168D	Australian/English	2	1	*Hinf*I
509T > C	3	F170S	Italian	4	2	*Mnl*I
516C > G	3	D172E	Italian	1	0	NA
573G > T	3	W191C	Italian	2	1	*Hae*III
615C > G	3	S205R	Italian	1	0	*Alu*I
49G > T	4a	G250V	Italian	1	0	NA
806-807insA	4a	N269fsX311	English	1	0	NA
863C > T	4a	R288W	French	2	1	NA
954-955insG	4a	G319fsX341	Italian	1	0	NA
959G > T	4b	G320V	Canadian/Italian	36	18	*Ban*I
1153C > T	4b	R385X	Italian	4	2	*Ava*I

NA indicates not applicable.

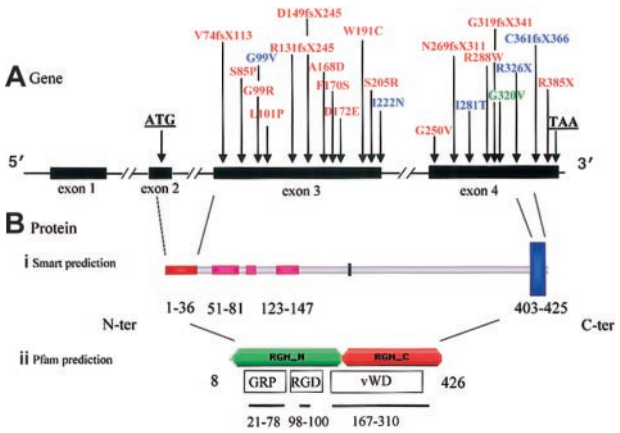

Figure 1. Molecular analysis of the hemojuvelin gene and protein. (A) Schematic representation of the genomic structure of the *HJV* gene. The position of the mutations is indicated above the gene. Novel mutations detected in this study are in red, mutations previously described[14] are in blue, mutations identified in both papers are in green. (B) Predicted domains of the hemojuvelin protein according to SMARTv4 (http://smart.embl-heidelberg.de) and Pfam (http://www.sanger.ac.uk/Software/Pfam) databases. The signal peptide is marked in red, the low-complexity regions are in pink, and the carboxy-terminal peptide is in blue. The region of homology to RGM, a glycosyl-phosphatidyl-inositol (GPI)–linked axon guidance molecule, the RGD motif, the von Willebrand–like domain, and the C-terminal transmembrane domain are indicated. GRP indicates glycine-rich protein.

are reported in Table 2. Figure 1A summarizes all known mutations, including those previously described.[14]

Most non-Canadian (13 of 17) and all French-Canadian patients were mutation homozygous, consistent with the high degree of consanguinity previously observed in this disorder.[11-13] Compound heterozygotes were rare in our series.

As shown in Table 2 and in Figure 1A, all mutations occur in exons 3 and 4. Six mutations result in premature termination codons. Homozygous R385X and D149fsX245 were found, respectively, in 2 and 3 apparently unrelated Italian probands. Two probands pairs, however, shared the same haplotype combination of marker alleles.[22] The homozygous 391 to 403 thirteen–bp (CGGGGCCCCGCCC) deletion, expected to produce a null phenotype, was found in a single Italian patient. The mechanism of nucleotide loss is likely related to a sequence repeat (CCCCGGCCCC) that favors strand misalignment and subsequent deletion. The 220delG mutation, producing a frameshift and a stop signal at codon 113, was detected in compound heterozygosity with 806-807insA, that also results in a frameshift and leads to truncation at position 311. This association, characterized in 2 siblings from the United Kingdom, is expected to produce a reduced amount of a truncated protein of 310 amino acids. R385X also results in a shorter peptide lacking the 42 carboxy-terminal residues that, based on homology programs, likely correspond to the putative transmembrane domain (Figure 1B).

Eleven mutations are missense. All French-Canadian patients homozygous for an identical haplotype[21] were homozygous for G320V, a previously reported mutation, reported to be common in a cohort of Greek patients with JH.[14] The same mutation was found in one patient from a Southern Italian village where a dialect resembling Greek is still spoken. F170S was found in 2 Italian patients with different 1q haplotypes. All other missense mutations were private and detected in single alleles/patients.

All amino acid replacements so far characterized occur at positions that are highly conserved in mouse and rat; all but A168 are conserved also in zebra fish (not shown). G99R affects the same RGD residue of a previously reported (G99V) mutation[14] but with a different amino acid substitution. S85P and L101P are close to the RGD motif. All the others take place in the von Willebrand–like motif, which spans amino acids 167 to 310 (Figure 1B).

Cosegregation with the disease phenotype was confirmed for all mutations through family studies by sequencing, restriction enzyme analysis (Table 2), or, in case of the 13-bp deletion, direct inspection of the PCR product on 2% agarose gel. Analysis by DHPLC of exon 3 and 4 amplicons excluded the presence of the identified mutations in 100 normal chromosomes.

Table 3. Genotype-phenotype correlation in patients

No.	Patients*	Sex	Age, y	TS, %	SF, μg/L	Cardiomyopathy	Hypogonadism	Liver biopsy histology	Patient/ family	*HJV* mutation	Reference
1	F1 II-1	F	15	88	615	−	−	Fb	2†	R385X	De Gobbi et al[10]
2	F2 II-1	F	20	82	1400	−	+	C	1	F170S	De Gobbi et al[10]
3	F3 II-1	F	21	84	3500	−	+	Fb	1	W191C	De Gobbi et al[10]
4	F4 II-2	M	20	ND	2000	−	+	—	1	R385X	De Gobbi et al[10]
5	F5 II-1‡	F	20	98	3768	+	+	C	1	D149fsX245	De Gobbi et al[10]
6	F7 II-2	F	21	75	2300	+	+	Fb	2	S205R/G250V	De Gobbi et al[10]
7	F8 II-5	F	14	92	3280	−	+	Fb	2	F170S	De Gobbi et al[10]
8	F9 II-2	F	24	100	2130	−	+	C	2	V74fsX113/N269fsX311	De Gobbi et al[10]
9	F10 II-2	M	21	100	2850	−	+	C	2	D149fsX245	De Gobbi et al[10]
10	F18 II-1	F	20	100	4840	+	+	—	1	R131fsX245	De Gobbi et al[10]
11	F19 II-1	M	29	100	5665	−	+	C	1	G320V	De Gobbi et al[10]
12		F	30	100	823	−	+	Fb	1	S85P	Unpublished
13		F	26	100	12 500	+	+	Fb	2†	R288W	Durand et al[23]
14		F	20	ND	2500	+	+	—	2†	D172E/G319fsX341	Unpublished
15		M	28	ND	—	−	+	—	3	A168D	Unpublished
16		F	26	100	2500	+	+	—	1	L101P/G99R	Unpublished
17		M	22	95	2800	−	+	Fb	2	D149fsX245	Unpublished
18	1057	F	27	100	> 2500	+	+	—		G320V	Rivard et al[21]

TS indicates transferrin saturation; SF, serum ferritin; ND, not done; Fb, fibrosis; C, cirrhosis; +, presence of complication; and −, absence of complication.
*Clinical data of the proband are shown for each family. A single patient is shown from the 12 related Canadian families.
†One subject died as a result of cardiac failure.
‡β-thalassemia trait.

The clinical phenotype of most of the patients here studied has already been published.[10,21] A summary of the clinical data related to the characterized mutations in patients is reported in Table 3.

Discussion

Our results show a striking allele heterogeneity of JH and reinforce the concept that mutations of the *HJV* gene are mainly private. We detected 16 novel deleterious nucleotide substitutions/deletions, thereby significantly expanding the spectrum of mutations in this gene. We confirm that G320V is a recurrent mutation in different populations,[14] which, because of founder effect and inbreeding, accounts for all cases of JH in Saguenay-Lac-St-Jean. However, most Italian patients have their own spectrum of mutations. The only G320V Italian homozygote is likely of Greek ancestry, because he was born in a restricted geographic area in Southern Italy, where Greek traditions and language are still alive.

D149fsX245, R385X, and F170S are the most common mutations in our series, but we cannot exclude that patients with the same mutation may be distantly related. On the basis of its proposed mechanism of formation, we speculate that the 13-bp deletion might be detected in other cases.

The large number of missense mutations marks residues/domains relevant for the protein function. Most mutants occur within the von Willebrand–like domain (Figure 1B). Three mutations (S85P, G99R, and L101P) cluster around the RGD domain (position 98-100). The occurrence of 2 changes at the same position (glycine 99) further strengthens the essential role of the RGD motif in the protein.

As shown in Table 3, the genotype-phenotype correlation is difficult to assess in the context of the large number of observed mutations. Considering only the genetically restricted group of Canadian patients, all carrying the same G320V mutation, still a phenotypic variation in terms of both age at diagnosis and organ damage is observed.

Beside allele heterogeneity JH is characterized by genetic heterogeneity, which provides indirect insights into the function of hemojuvelin that is not yet defined in humans or in mice. Both hemojuvelin and hepcidin mutations[15] cause an identical phenotype, indicating that both proteins cooperate to restrict iron absorption in the gut and that, in keeping with their evolutionary conservation, their function is not redundant. Two findings are inconsistent with the possibility that hemojuvelin represents a receptor for hepcidin. First, *HJV* RNA is prevalently expressed in the same tissues that express high concentrations of hepcidin (liver, skeletal muscle, and heart) and not in tissues that are considered the target of the hepcidin effect (duodenum and spleen macrophages)[14] (A.R. and C.C., unpublished data, January 9, 2004). Second, urinary hepcidin excretion is low in patients with juvenile hemochromatosis caused by mutations at the 1q locus,[14] indicating that mutant hemojuvelin has an inhibitory effect on hepcidin expression. From these findings it seems more likely that hemojuvelin is a component of the signaling pathway which activates hepcidin.

The results here presented, together with previous reports,[14,16] provide information on the proportion of hemojuvelin- and hepcidin-related JH, an issue which is relevant to the definitive diagnosis of iron overload in severely affected young patients by the molecular analysis of these human genes. Hepcidin mutations have been so far documented only in 4 families.[15-16,24] From the data at present available hemojuvelin mutations account for all the other patients with JH[14] (present paper). Inability to appropriately increase hepcidin with iron loading is the pathogenetic clue to *HFE*-related hemochromatosis, both in humans[25,26] and in animal models.[27,28] It has recently been shown that $Hfe^{-/-}$ mice that lack a single hepcidin allele have greater liver iron accumulation than mice lacking Hfe as a result of targeted gene disruption, indicating that hepcidin may serve as a modifier of *HFE* disease.[29] However, hepcidin mutations are extremely rare among patients with *HFE* C282Y.[30-32] In the light of these findings and the putative functional link between hepcidin and hemojuvelin, we believe that there is a case for exploring the potential role of variant hemojuvelin alleles in the clinical expression of human iron overload with particular reference to *HFE* hemochromatosis.

Acknowledgments

We thank Doria Grimard, Raynald Simard, Mark de Braekeleer, Maryse Beaudet, and Diane Bourassa for their support. We also thank Susan Manass, Antonio Tabilio, and F. Di Vito for referring samples of patients. We thank TIGEM Mutation Detection Core and TIGEM Sequencing Core for their technical assistance.

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