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

Carmela Lanzara, Antonella Roetto, Filomena Daraio, Silvain Rivard, Romina Ficarella, Hervey Simard, Timothy M. Cox, Mario Cazzola, Alberto Piperno, Anne-Paule Gimenez-Roqueplo, Paola Grammatico, Stefano Volinia, Paolo Gasparini, and Clara Camaschella

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) © 2004 by The American Society of Hematology

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. 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. All these features are consistent with a mild effect of HFE 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. Compared with HFE-relatedand transferrin receptor 2–related or type 3 hemochromatosis, the clinical course of JH is rapid and severe, indicating that the JH protein is an important regulator of iron absorption. JH is genetically heterogeneous because it is associated with 2 distinct chromosomal loci. Most patients have a disorder linked to 1q. The 1q locus was mapped in 1999 in families of Italian descent, but the cognate gene remained elusive until recently. 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. Hepadin encodes the key regulator of iron absorption in mice, 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.

The JH gene that maps to 1q was recently identified through an elegant positional cloning approach. 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. The nomenclature of the different hemochromatosis types is controversial. We used the term “JHV” (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, an RGD motif, and a von Willebrand–like domain. One frameshift, one nonsense, and 4 missense mutations in highly conserved residues proved the causality of the gene in juvenile hemochromatosis. 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.
Table 1. Sequences of the primers used for screening of HJV mutations

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>PCR product length, bp</th>
</tr>
</thead>
<tbody>
<tr>
<td>Exon 1 F</td>
<td>5’ CCGTCAGTCTGGTGGCC 3’</td>
<td>336</td>
</tr>
<tr>
<td>Exon 1 R</td>
<td>5’ CATTTGACAGAGACACTC 3’</td>
<td></td>
</tr>
<tr>
<td>Exon 2 F</td>
<td>5’ GCCAATTCGACGTTGCTT 3’</td>
<td>380</td>
</tr>
<tr>
<td>Exon 2 R</td>
<td>5’ CTTATCCAGGCATTGCTGC 3’</td>
<td></td>
</tr>
<tr>
<td>Exon 3 F</td>
<td>5’ CTGCAAGAGCAACAGGTGCTG 3’</td>
<td>709</td>
</tr>
<tr>
<td>Exon 3 R</td>
<td>5’ GGACTTCTGCTGATTGCTTC 3’</td>
<td></td>
</tr>
<tr>
<td>Exon 4a F</td>
<td>5’ AACTGTGTAGGAGGCTGTTG 3’</td>
<td>840</td>
</tr>
<tr>
<td>Exon 4a R</td>
<td>5’ CTGAGGCTCTGTGGTACTC 3’</td>
<td></td>
</tr>
<tr>
<td>Exon 4b F</td>
<td>5’ CAGCTGACAGACGCTGC 3’</td>
<td>1043</td>
</tr>
<tr>
<td>Exon 4b R</td>
<td>5’ CACATTGCTTATTTACTCATTTC 3’</td>
<td></td>
</tr>
</tbody>
</table>

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, and mutations in hemicidin were excluded. Seventeen French-Canadian affected individuals were from 12 families of the isolated region of Saguenay-Lac-Saint Jean (Quebec) and were previously shown to have a disorder in linkage with the 1q locus. All patients included in the study had a clinical diagnosis of JH according to the proposed criteria.

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 MgCl2 (15 mM), 2 μL dNTP (deoxyribonucleotide triphosphate; 2.5 mM), 0.5 μL each primer (20 pM/L), 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.

Polymers at chain reactions 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 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 by 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 Turin 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
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 (CGGGGCCCCGCC) 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 (CCCCGCCC-C) 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 haplotype21 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) mutation14 but with a different amino acid substitution. S85P and L101P are close to the RGD motif, the von Willebrand–like domain, and the C-terminal transmembrane domain are indicated. GRP indicates glycine-rich protein.

Table 3. Genotype-phenotype correlation in patients

<table>
<thead>
<tr>
<th>No.</th>
<th>Patients*</th>
<th>Sex</th>
<th>Age, y</th>
<th>TSI, %</th>
<th>SF, μg/L</th>
<th>Cardiomyopathy</th>
<th>Hypogonadism</th>
<th>Liver biopsy histology</th>
<th>Patient/ family</th>
<th>HJV mutation</th>
<th>Reference</th>
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<tbody>
<tr>
<td>1</td>
<td>F1 II-1</td>
<td>F</td>
<td>15</td>
<td>88</td>
<td>615</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>Fb 2†</td>
<td>R385X</td>
<td>De Gobbi et al10</td>
</tr>
<tr>
<td>2</td>
<td>F2 II-1</td>
<td>F</td>
<td>20</td>
<td>82</td>
<td>1400</td>
<td>–</td>
<td>+</td>
<td>C</td>
<td>1 F170S</td>
<td>De Gobbi et al10</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>F3 II-1</td>
<td>F</td>
<td>21</td>
<td>84</td>
<td>3500</td>
<td>–</td>
<td>+</td>
<td>Fb 1</td>
<td>W191C</td>
<td>De Gobbi et al10</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>F4 II-2</td>
<td>M</td>
<td>20</td>
<td>ND</td>
<td>2000</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>1 R385X</td>
<td>De Gobbi et al10</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>F5 II-‡</td>
<td>F</td>
<td>20</td>
<td>98</td>
<td>3768</td>
<td>+</td>
<td>–</td>
<td>C</td>
<td>1 D149fsX245</td>
<td>De Gobbi et al10</td>
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<tr>
<td>6</td>
<td>F7 II-2</td>
<td>F</td>
<td>21</td>
<td>75</td>
<td>2300</td>
<td>+</td>
<td>–</td>
<td>Fb 2</td>
<td>S205R/G250V</td>
<td>De Gobbi et al10</td>
<td></td>
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<tr>
<td>7</td>
<td>F8 II-5</td>
<td>F</td>
<td>14</td>
<td>92</td>
<td>3280</td>
<td>–</td>
<td>+</td>
<td>Fb 2</td>
<td>F170S</td>
<td>De Gobbi et al10</td>
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<tr>
<td>8</td>
<td>F9 II-2</td>
<td>F</td>
<td>24</td>
<td>100</td>
<td>2130</td>
<td>–</td>
<td>+</td>
<td>C</td>
<td>2 V74fsX113/N269fsX311</td>
<td>De Gobbi et al10</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>F10 II-2</td>
<td>M</td>
<td>21</td>
<td>100</td>
<td>2850</td>
<td>–</td>
<td>+</td>
<td>C</td>
<td>2 D149fsX245</td>
<td>De Gobbi et al10</td>
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<tr>
<td>10</td>
<td>F18 II-1</td>
<td>F</td>
<td>20</td>
<td>100</td>
<td>4840</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>1 R131fsX245</td>
<td>De Gobbi et al10</td>
<td></td>
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<tr>
<td>11</td>
<td>F19 II-1</td>
<td>M</td>
<td>29</td>
<td>100</td>
<td>5665</td>
<td>+</td>
<td>–</td>
<td>C</td>
<td>1 G320V</td>
<td>De Gobbi et al10</td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>F3 II</td>
<td>F</td>
<td>30</td>
<td>100</td>
<td>823</td>
<td>–</td>
<td>+</td>
<td>Fb 1</td>
<td>S85P</td>
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<tr>
<td>13</td>
<td>F3 II</td>
<td>F</td>
<td>26</td>
<td>100</td>
<td>12,500</td>
<td>+</td>
<td>+</td>
<td>Fb 2†</td>
<td>R288W</td>
<td>Durand et al15</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>F2 II</td>
<td>F</td>
<td>20</td>
<td>ND</td>
<td>2500</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>2† D172E/G319fsX341</td>
<td>Unpublished</td>
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</tr>
<tr>
<td>15</td>
<td>M II-8</td>
<td>M</td>
<td>28</td>
<td>ND</td>
<td>2500</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>3 A168D</td>
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<td></td>
</tr>
<tr>
<td>16</td>
<td>F1 II</td>
<td>F</td>
<td>26</td>
<td>100</td>
<td>2500</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>1 L101P/G99R</td>
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<tr>
<td>17</td>
<td>M II-10</td>
<td>M</td>
<td>22</td>
<td>95</td>
<td>2800</td>
<td>–</td>
<td>+</td>
<td>Fb 2</td>
<td>D149fsX245</td>
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<td></td>
</tr>
<tr>
<td>18</td>
<td>1057</td>
<td>F</td>
<td>27</td>
<td>100</td>
<td>&gt;2500</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>G320V</td>
<td>Rivard et al11</td>
<td></td>
</tr>
</tbody>
</table>

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. 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, 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 mutations 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 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), 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, 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. From the data at present available hemojuvelin mutations account for all the other patients with JH (present paper). Inability to appropriately increase hepcidin with iron loading is the pathogenetic clue to HFE-related hemochromatosis, both in humans and in animal models. It has recently been shown that Hfe-deficient 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 modulator of HFE disease. However, hepcidin mutations are extremely rare among patients with HFE C282Y. 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

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References


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