Global Sequencing Approach for Characterizing the Molecular Background of Hereditary Iron Disorders

Séverine Cunat,1 Muriel Giansily-Blaizot,1 Michael Bismuth,2 François Blanc,3 Olivier Dereure,4 Dominique Larrey,2 Alain Le Quellec,5 Philippe Pouderoux,6 Christian Rose,7 Isabelle Raingeard,8 Eric Renard,8 Jean-François Schved,1 Patricia Aguilar-Martinez,1* and the CHU Montpellier AOI 2004 Working Group

Background: New genetic forms of hereditary hemochromatosis (HH) or hereditary hyperferritinemia (HF) have been identified over the last few years, and abnormalities of various genes may interact in a single patient. This study aimed to develop a rapid automated method for sequencing the main genes involved.

Methods: We used a standard 96-well microplate with a single PCR condition in an adaptation of the SCAIP (single-condition amplification with internal primer) method to sequence the HFE (hemochromatosis), HAMP (hepcidin antimicrobial peptide), HFE2/HJV [hemochromatosis type 2 (juvenile)], SLC40A1 (ferroportin), and TFR2 (transferrin receptor 2) genes, and the 5′ untranslated region of the FTL (ferritin, light polypeptide) gene. To further simplify the method, we adjusted PCR conditions to avoid the use of an internal primer and applied this single-condition amplification method to 38 selected, unrelated patients. We tailored the genetic investigation according to the clinical picture, with the patients falling into 2 groups. Group 1 consisted of patients with hyperferritinemia and high transferrin saturation (TS) (classic adult and juvenile HH forms, groups 1A and 1B, respectively), and group 2 consisted of patients with hyperferritinemia and low, typical, or slightly increased TS, with or without iron overload (groups 2A and 2B, respectively).

Results: With this strategy we identified single-gene and multigene abnormalities, including 6 previously undescribed abnormalities in HFE (c.794dupA), HFE2 (c.–89–4dupT), and SLC40A1 (c.262A > G, c.533G > A, c.1468G > A, and c.–59_–45del).

Conclusion: This method is a simple approach for investigating hereditary iron overload or HF and allows rapid evaluation of patients.

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Hereditary hemochromatosis (HH)9 is the main cause of inherited iron overload. When present in a homozygous state, the Cys282Tyr mutation in the hemochromatosis (HFE)10 gene product is the most common genetic background of HH (1). Ten years after the description of this genotype, further investigations of the disease have revealed considerable heterogeneity.

First, other HFE genotypes, including the compound heterozygote encoding the Cys282Tyr and His63Asp mutations, have been associated with an HH phenotype, although individuals with such genotypes accumulate less iron than Cys282Tyr homozygotes (1–3). Other rare private HFE mutations responsible for HH have also been reported. Second, Cys282Tyr homozygotes have been demonstrated to lack full penetrance (4), and some indi-
viduals homozygous for the Cys282Tyr mutant remain asymptomatic throughout life. Investigators have also described several other genes that present a phenotype similar to that of HFE-related HH, indicating that hemochromatosis is a multigene disorder. Nowadays, HH is considered an autosomal-recessive inherited disorder with 2 clinical forms, adult and juvenile HH (5, 6), defined according to the age of onset. Juvenile hemochromatosis (JH) is due to a mutation in 1 of 2 genes: HFE2 [hemochromatosis type 2 (juvenile); also known as HJV and encoding hemouvelin] (7) and, more rarely, HAMP (hepcidin antimicrobial peptide) (8). Adult HH is mainly due to mutation in HFE, and TFR2 (transferrin receptor 2) mutations are rarely found (9). Associations between mutations of different genes leading to modified clinical presentations have been described for some patients. Such findings demonstrate that hemochromatosis can be a multigenic or at least a digenic disease. Indeed, HAMP (10), HFE2 (11), or TFR2 (12) mutations that have been associated with HFE genotypes have been described to aggravate the clinical presentation.

On the other hand, clinical pictures characterized by high ferritinemia have recently emerged [for review, see (13)]. Hereditary hyperferritinemia (HF) mostly has a dominant inheritance pattern and may or may not feature iron overload. No iron overload is associated with hered-
were named in accordance with the standard international nomenclature guidelines recommended by the Human Genome Variation Society (HGVS at http://www.hgvs.org/mutnomen/recs.html).

**SINGLE-CONDITION AMPLIFICATION METHOD**
We extracted DNA from patient’s peripheral blood leukocytes with standard procedures. Direct sequence analysis of all the targeted iron metabolism-related genes was based on the SCAIP method (19), which allows simultaneous amplification, purification, and sequence analysis of multiple DNA regions on a standardized microplate with a single PCR condition. We used a simplified version of this method, the single-condition amplification (SCA) method, by adjusting PCR conditions according to Roux et al. (20), thereby avoiding the need for an internal primer. We chose amplicons to cover the 39 exons, exon/intron boundaries, and most of the 5’ and 3’ UTRs of HFE, TFR2, HFE2, HAMP, and SLC40A1, as well as the 5’ UTR of FTL. This set constituted 44 amplicons spanning 16.4 kb over a total of 64.8 kb of genomic DNA sequence. Primer sets were adapted from those described in previously published articles or were designed for this study (see Table 1 in the Data Supplement that accompanies the online version of this article at http://www.clinchem.org/content/vol53/issue12). Each amplicon was designed for an optimal size range of 200–500 bp to maintain uniform PCR conditions and tested for compatibility under temperature “stepdown” conditions (21), an adaptation of the “touchdown” PCR method (22). The appropriate amplification gradient used for each gene is given in Table 2 in the online Data Supplement.

**DNA SEQUENCING**
Sequencing reactions were carried out in a 10-µL volume containing 10–50 fmol PCR template, 1.5 µL 5× BigDye® Terminator v1.1 Sequencing Buffer (Applera), 5 pmol/µL sequencing primer, 1.5 µL ABI PRISM® BigDye Terminator v1.1 reaction mixture (Applera), and 4 µL sterile water. Sequencing products were then purified on Montage®-SEQ96 microplates (Millipore) according to the manufacturer’s instructions. We added 15 µL of Hi-Di® formamide (Applera) to each well as recommended (22) and electrophoresed the samples on an ABI PRISM 310 DNA analyzer (Applera). We then analyzed sequence-trace files with Sequencing Analysis Software (version 5.1.1; Applera) and used SeqScape® software (version 2.1.1; Applera) to compare the data with the GenBank reference sequence.

**CONTROL OF DNA SEQUENCING**
DNA from an anonymous control individual with typical erythrocytes and nonpathologic values for iron variables was sequenced with the SCA method in parallel with a classic sequencing method for all of the studied genes (23). For internal positive controls, we used samples that our group or others had previously identified to possess mutations.

**ANALYSES OF INDIVIDUAL MUTATIONS AND OF PARENTS OR SIBLINGS**
We confirmed an identified mutation using a novel PCR product either by restriction enzyme analysis when available or by another sequencing experiment. We carried out a segregation analysis of family members of patients with an identified mutation.

**Results**
THE SCA SEQUENCING METHOD ADAPTED TO IRON METABOLISM-RELATED GENES
Successive adaptations, including primer choice, stepdown PCR conditions, and purification conditions, were necessary to permit easy screening of the 6 targeted genes related to iron metabolism. We optimized the conditions to apply the method to the 2 main phenotypic profiles: HH/group 1 (HFE, TFR2, HFE2, and HAMP) or HF/group 2 (SLC40A1 and FTL). The TFR2 gene had to be processed separately because the annealing temperatures for TFR2 were substantially higher than for the other HH genes (see Table 2 in the online Data Supplement). The sample requirements for a maximum of 44 amplicons per patient are relatively low, approximately 44 µg DNA. One cycle-sequencing plate can be used for a complete study of the different genes for a single patient or to screen a given gene for different patients. The implementation of the SCA technique in our laboratory has allowed us to greatly shorten the time required to complete a diagnosis of iron-related disorders. Generally, only 1 week is necessary to complete the sequencing steps for a 96-well plate, including analysis of the sequencing results. If a gene defect is identified, further investigation is needed to confirm the mutation.

**APPLICATION TO THE IDENTIFICATION OF NOVEL MUTATIONS IN GENES RELATED TO IRON METABOLISM**
We studied 38 patients with this method, including 16 patients with a suspected diagnosis of HH and 22 patients with suspected HF. The patients with potentially causative mutations are described in Table 1. In addition, we identified several previously known or undescribed polymorphisms during the course of the study (see Table 3 in the online Data Supplement) and found no mutation in the targeted genes for 29 of the patients.

In group 1, 1 patient had a new HFE mutation, 1 patient had a novel HFE2 mutation, and 2 patients had a new SLC40A1 mutation. The new HFE mutant was a frameshift mutation, c.794dupA, in HFE exon 4 of patient HG3572 and was in trans of the Cys282Tyr mutant (see Fig. 1 in the online Data Supplement). This patient was classified into group 1A (classic HH). The HFE2 mutation was found in patient HG2280, a 23-year-old woman referred for discrepancies between her HFE genotype and the clinical phenotype. Although she was homozygous for
the Cys282Tyr mutation and had a classic adult form of HH, as did her mother, the patient had abnormally high values of iron-related variables for her age (group 1B). A heterozygous T insertion 5’ to exon 2 (c.–89→4dupT) was identified in the HFE2 gene inherited from the father, who was a Cys282Tyr/His63Asp compound heterozygote and affected with an adult form of HH. This insertion was not present in the mother. The HFE, HAMP, and TFR2 genes were wild type. A final assignment of causative status for this mutation will require analysis of HFE2 transcripts. Patient HG3943, age 73 years, was referred for suspected classic HH because of a high ferritin concentration (1500 μg/L) and high TS (80%) (group 1A). The HFE genotype was simply a His63Asp heterozygote. An MRI examination of the liver revealed iron overload with a hepatic iron concentration of 240 μmol/g. The patient also had liver enzyme anomalies. The genes involved in HH, namely HFE, TFR2, HFE2, and HAMP, were wild type. An undescribed short deletion, c.59–45del, was identified in the 5’ UTR of the SLC40A1 gene. The 2nd patient, HG3135, is described below.

Group 2 included 2 unrelated 58-year-old female patients who were referred for isolated hyperferritinemia without iron overload and with cataracts (Table 1, group 2B). Both patients had a previously described mutation in the 5’ UTR of the FTL gene. Three unrelated patients had novel heterozygous mutations in the SLC40A1 gene (Table 1, group 2A).

Patient HG3181, a 38-year-old man, was referred for high hyperferritinemia with slightly increased TS and a family history of iron overload. He had a wild-type HFE gene, and the genes involved in HH were also wild type. Analysis of the SLC40A1 gene revealed a missense mutation (c.262A>G) in exon 3 leading to an Arg88Gly substitution. This modification was reported simultaneously by us and another group (Aguilar-Martinez et al., unpublished data; Jouanolle et al., personal communication, January 30, 2004). It is noteworthy that the patient had become anemic under a phlebotomy program, which therefore had to be stopped. A liver biopsy performed at another center, which was initially unavailable to us, recently confirmed the iron overload in mixed parenchymal and Kupffer cells typically seen in “ferroportin disease”. The patient’s father, who had the same mutation, recently died of liver cancer.

Two apparently unrelated patients (HG3142 and HG3134) who were referred independently shared the same previously undescribed c.1468G>A nucleotide substitution in SLC40A1. This mutation produces a Gly490Ser substitution at the same position of another substitution, Gly490Asp, which has previously been described to lead to ferroportin disease (24). Patient HG3142, who at 24 years at the time of diagnosis was the youngest of both, had isolated hyperferritinemia with typical TS and no additional biochemical abnormality, including hepatic enzymes (group 2A). The 2nd patient (HG3134) was a 71-year-old woman who had high hyperferritinemia (2710 μg/L) associated with high TS (83%). She experienced chronic fatigue and a cataract and was heterozygous for the His63Asp substitution. An MRI evaluation demonstrated a heavy hepatic iron overload (350 μmol/g). The FTL gene was wild type. This patient’s phenotype placed her in group 1A. The apparent phenotypic discrepancy between these 2 patients with the same mutation is discussed below.

Finally, patient HG4421 had a personal and family history of hyperferritinemia. She had a typical TS and no common HFE mutation and was classified in group 2A. A new mutation in the SLC40A1 gene (c.533G>A) was in heterozygous condition (Table 1).

**Discussion**

Clinical genetic testing in iron-overload disorders routinely focuses on the detection of the Cys282Tyr and His63Asp mutations encoded by the HFE gene. Recently, investigators have recognized the need to expand mutational investigation to other genes associated with iron overload. Sequencing analysis via the classic methods of sequencing individual samples is time-consuming because of the number and, in some cases, the size of the iron metabolism-related genes (TFR2 has 18 exons) and because such genes have to be studied with a variety of annealing and PCR conditions. Consequently, such strategies are considered labor intensive and expensive. A limited number of other global approaches, mainly involving denaturing HPLC, for the screening of mutations in genes involved in iron metabolism have been proposed in the literature (10, 25). Other DNA-screening methods, such as microelectronic DNA chips (26), have been applied to the study of single iron-related genes. These methods, although useful, require expertise to set up the analysis for each individual gene. They also may have limitations in sensitivity, and some molecular defects may be missed. Another drawback is that these approaches are screening methods, and a subsequent sequencing step is needed to precisely identify the sequence variation and to distinguish known and unknown polymorphisms from deleterious mutations. We have described an efficient and simple sequencing method (SCA) for the molecular diagnosis of HH or HF that we have adapted from the SCAIP technique of Flanigan et al. (19). This method, which is based on a SCA followed by gene sequencing, permits the amplification of large numbers of ampiclons at a determined set of PCR temperatures. We used stepdown PCR conditions, an approach derived from touchdown PCR (22) that conveniently bypasses spurious amplification (21, 27). This strategy makes primer choice easier, because the melting temperature of each primer can be situated in an area determined by the stepdown conditions. In our case, the melting temperature was chosen to permit the simultaneous amplification of iron metabolism–related genes that we clustered according to 3 specified clinical profiles.
Table 1. Main clinical, biochemical, histological, and genotypic data of the patients.

<table>
<thead>
<tr>
<th>Group 1 (hyperferritinemia with high TS and parenchymal iron overload)</th>
<th>Group 1B (juvenile form of HH)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Patient no.</strong></td>
<td>HG3572</td>
</tr>
<tr>
<td><strong>Sex</strong></td>
<td>Male</td>
</tr>
<tr>
<td><strong>Age at molecular diagnosis, years</strong></td>
<td>47</td>
</tr>
<tr>
<td><strong>Hepatopathy</strong></td>
<td>No</td>
</tr>
<tr>
<td><strong>Hypogonadotropic hypogonadism</strong></td>
<td>No</td>
</tr>
<tr>
<td><strong>Other clinical signs</strong></td>
<td>No</td>
</tr>
<tr>
<td><strong>Serum iron, µmol/L</strong></td>
<td>NA</td>
</tr>
<tr>
<td><strong>Serum ferritin, g/L</strong></td>
<td>1007</td>
</tr>
<tr>
<td><strong>TS, %</strong></td>
<td>69</td>
</tr>
<tr>
<td><strong>HIC, µmol/g</strong></td>
<td>140</td>
</tr>
<tr>
<td><strong>HIC/age, µmol/g/years</strong></td>
<td>3.0</td>
</tr>
</tbody>
</table>

| **HFE** | | | | | |
| --- | | | | | |
| p.Cys282Tyr | CY | CC | CC | YY | CC |
| p.His63Asp | HH | HD | HD | HH | DD |
| Other HFE mutation | c.794dupA (p.Trp267LeufsX80) | No | No | No | No |
| HFE polymorphism | No | ND | c.[340+4T>C]+[=] | No | No |
| TFR2 | c.[1851C>T]+[=] (p.Ala617) | ND | c.[=]+[=] | c.[=]+[=] | c.[=]+[=] |
| HAMP | c.[=]+[=] | ND | c.[=]+[=] | c.[=]+[=] | c.[=]+[=] |
| HFE2 | c.[=]+[=] | ND | c.[=]+[=] | c.[=]+[=] | c.[=]+[=] |
| SLC40A1 | c.[=]+[=] | c.[1468G>A]+[=] | c.[=]+[=] | c.[=]+[=] | c.[=]+[=] |
| FTL, 5’ UTR | ND | c.[=]+[=] | ND | ND | ND |
**Table 1. Continued**

<table>
<thead>
<tr>
<th>Group 2 (hyperferritinemia with typical, low, or slightly increased TS)</th>
<th>Group 2A (evidence of tissue iron overload)</th>
<th>Group 2B (cataract, no tissue iron overload)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Patient no.</td>
<td>HG3142</td>
<td>HG3181</td>
</tr>
<tr>
<td>Sex</td>
<td>Male</td>
<td>Male</td>
</tr>
<tr>
<td>Age at molecular diagnosis, years</td>
<td>24</td>
<td>41</td>
</tr>
<tr>
<td>Hepatopathy</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Hypogonadotrophic hypogonadism</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Cardiomyopathy</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Other clinical signs</td>
<td>Chronic fatigue, joint pain</td>
<td>No</td>
</tr>
<tr>
<td>Serum iron, μmol/L</td>
<td>17.5</td>
<td>6.2</td>
</tr>
<tr>
<td>Serum ferritin, g/L</td>
<td>1262</td>
<td>2200</td>
</tr>
<tr>
<td>TS, %</td>
<td>32</td>
<td>7.1, after phlebotomies</td>
</tr>
<tr>
<td>HIC, μmol/g</td>
<td>NA</td>
<td>65</td>
</tr>
<tr>
<td>HIC/age, μmol/g/years</td>
<td>NA</td>
<td>1.6</td>
</tr>
<tr>
<td>HFE</td>
<td>p.Cys282Tyr</td>
<td>CC</td>
</tr>
<tr>
<td></td>
<td>p.His63Asp</td>
<td>HH</td>
</tr>
<tr>
<td>Other HFE mutation</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>HFE polymorphism</td>
<td>ND</td>
<td>c.[892+48G&gt;A]+[=]</td>
</tr>
<tr>
<td>TFR2</td>
<td>c.[262A&gt;G]+[=]</td>
<td>c.[=]+[=]</td>
</tr>
<tr>
<td>HAMP</td>
<td>c.[=]+[=]</td>
<td>c.[=]+[=]</td>
</tr>
<tr>
<td>HFE2</td>
<td>c.[=]+[=]</td>
<td>c.[=]+[=]</td>
</tr>
<tr>
<td>SLC40A1</td>
<td>c.[1468G&gt;A]+[=]</td>
<td>c.[=]+[=]</td>
</tr>
<tr>
<td>FTL, 5’ UTR</td>
<td>c.[=]+[=]</td>
<td>c.[=]+[=]</td>
</tr>
</tbody>
</table>

*a* Hepatic iron concentration; CY, Cys282/Tyr282 heterozygote; CC, Cys282/Cys282 homozygote; YY, Tyr282/Tyr282 homozygote; HH, His63/His63 homozygote; HD, His63/Asp63 heterozygote; DD, Asp63/Asp63 homozygote; ND, not done; NA, not available.

*a* Known HFE polymorphism in *HFE* intervening sequence (intron) 2 (IVS2) (39).

*b* Kasson polymorphism in *HFE* intervening sequence (intron) 2 (IVS2) (39).

A rare polymorphism of the *TFR2* gene in exon 17 (2085G>C) does not produce an amino acid change at residue 695 (Ser695). This sequence modification occurred in a young girl with JH caused by compound heterozygosity for *HFE2* mutations (36). This individual was included as a control in this study. It is unlikely this nucleotide change played a role in the increased values for iron-related variables in this girl, because this change was also present in her father, who had typical values for serum iron indices. This nucleotide substitution was not found in any of the other tested patients.

*c* Known *HFE* polymorphism (40). For details on the polymorphisms identified in this study, see Table 3 in the online Data Supplement.
Table 2. Arguments for a causative role for the newly identified mutants.

<table>
<thead>
<tr>
<th>Gene</th>
<th>Mutation type</th>
<th>Location (gene)</th>
<th>Protein modification</th>
<th>Associated mutation in iron-related gene (HFE, HAMP, HFE2, SLC40A1)</th>
<th>Study of 100 control chromosomes</th>
<th>Patient no./positive segregation for family</th>
<th>Genotype-phenotype correlation</th>
</tr>
</thead>
<tbody>
<tr>
<td>HFE</td>
<td>c.794dupA (heterozygous frameshift)</td>
<td>Exon 4</td>
<td>p.Trp267LeufsX80 (frameshift mutation creating a premature stop codon)</td>
<td>HFE (p.Cys282Tyr homozygote)</td>
<td>Absent</td>
<td>HG3572/Daughter (age 20 years) asymptomatic</td>
<td>Yes</td>
</tr>
<tr>
<td>HFE2</td>
<td>c.–89–4dupT (heterozygous intronic point mutation)</td>
<td>5' exon 2 (IVS1&lt;sup&gt;a&lt;/sup&gt; splicing site?)</td>
<td>No&lt;sup&gt;b&lt;/sup&gt;</td>
<td>HFE (p.Cys282Tyr homozygote)</td>
<td>Absent</td>
<td>HG2280/Mother; HFE, p.Cys282Tyr homozygote (classic HH adult form); HFE2, wild type; Father: HFE, p.Cys282Tyr and p.His63Asp compound heterozygote; HFE2, c.–89–4dupT heterozygote (classic HH adult form)</td>
<td>Yes</td>
</tr>
<tr>
<td>SLC40A1</td>
<td>c.1468G&gt;A (heterozygous missense)</td>
<td>Exon 8</td>
<td>p.Gly490Ser (another substitution, p.Gly490Asp, is known at the same position) (24)</td>
<td>No</td>
<td>Absent</td>
<td>Observed in 2 unrelated individuals (HG3134 and HG3142)/ NA</td>
<td>Yes</td>
</tr>
<tr>
<td>SLC40A1</td>
<td>c.533G&gt;A (heterozygous missense)</td>
<td>Exon 6</td>
<td>p.Arg178Gln</td>
<td>ND</td>
<td>Absent</td>
<td>HG4421/NA</td>
<td>Yes</td>
</tr>
<tr>
<td>SLC40A1</td>
<td>c.262A&gt;G (heterozygous missense)</td>
<td>Exon 3</td>
<td>p.Arg88Gly</td>
<td>No</td>
<td>Absent</td>
<td>HG3181/Father and paternal uncle heterozygous and clinically affected</td>
<td>Yes</td>
</tr>
<tr>
<td>SLC40A1</td>
<td>c.–59_–45del (heterozygous short deletion)</td>
<td>5' UTR (del 15 bp)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>No&lt;sup&gt;b&lt;/sup&gt;</td>
<td>No</td>
<td>Absent</td>
<td>HG3943/NA</td>
<td>Yes</td>
</tr>
</tbody>
</table>

<sup>a</sup> IVS1, intervening sequence (intron) 1; NA, not available; ND, not done.

<sup>b</sup> RNA analysis required to ascertain the causative role.

<sup>c</sup> In silico modification of transcription factor binding sites (loss of E4 and myogenin site, new Adf1 (alcohol dehydrogenase distal factor 1) site) with Alibaba 2.1 software (http://darwin.nmsu.edu/~molb470/fall2003/Projects/soorz/aliBaba_2_1.htm) (41).
Although the technical steps have been greatly simplified, reading this large number of sequences still remains a time-consuming step, even with the help of automated computer programs. The SCA is an interesting method for mutation analysis, but, like other sequencing-based technologies, its usefulness is limited for detecting gross rearrangements (deletions or duplications). Although this kind of lesion does not seem frequent in genes related to iron metabolism, specific testing of these defects could be performed in combination with the SCA method, as, for example, with multiplex amplifiable probe hybridization analysis of duplications (28), multiplex ligation-dependent probe amplification (29), or quantitative multiplex PCR of short fragments (30). We have planned to set up such methods to screen gross rearrangements of iron-related genes, especially in patients for whom SCA sequencing has identified no causative mutation. Indeed, by applying the SCA method to genes involved in iron metabolism, we were able to identify 9 potentially causative mutations among 38 patients with suspected HH or HF. The absence of detectable mutations in the remaining patients has not yet been explained. One possibility is that these patients have an unrecognized acquired condition. The correct phenotypic assessment of patients with increased ferritin concentrations or even with increased ferritin concentration and TS is often not complete before the patient is referred to the genetics laboratory. The establishment of rules could avoid unnecessary sequencing.

Unlike the use of such screening strategies as single-strand conformation polymorphism analysis, denaturing gradient gel electrophoresis, or denaturing HPLC, the SCA method permits immediate identification of a sequence variant. Known polymorphisms can be readily recognized so that rare mutations can be considered, depending on the position in the gene sequence and the mutation’s possible role in causing the phenotype. We used an extensive literature review and the criteria proposed by Cotton and Scriver (31) to ascertain the deleterious role of newly identified mutations (Table 2). Apart from 2 patients with previously identified FTL mutations, the other patients had new undescribed variations in iron metabolism-related genes.

Our attempt to make phenotype-to-genotype relationships among the identified defects stimulated interesting lines of discussion. We can outline 3 primary situations. First, digenism for mutations in other iron-related genes has been described for patients heterozygous for HFE Cys282Tyr mutation and an iron overload compatible with a diagnosis of adult HH (9–11, 32); however, the HFE gene must initially be screened because additional private mutations can be found in these patients (32–34). This is illustrated by patient HG3572, who had a 2nd frameshift mutation in the other HFE allele (Tables 1 and 2). Second, digenism involving HFE2, HAMP, or TFR2 mutations must be investigated in patients homozygous for the HFE Cys282Tyr substitution who have an abnormally severe or early clinical presentation, as was previously described. HFE2 seems to be the more frequently affected gene (9), and although additional studies are needed to ascertain the causative role of this mutation, this particular situation seems to be the case for patient HG2280 (Tables 1 and 2).

Finally, some patients in our series with ferroportin mutations initially had diagnoses of classic HH (group 1A patients HG3134 and HG3943; Table 1). The absence of mutations in HH genes led to the correct diagnosis. This finding is in keeping with the existence of 2 kinds of clinical profiles associated with different types of ferroportin mutations [for review, see (35)]. The 1st class of mutations leads to the originally described ferroportin disease, and the 2nd class displays a phenotype indistinguishable from HH. These 2 classes can be difficult to separate by only phenotype, however, especially in older patients (e.g., patients HG3134 and HG3943, ages 71 and 73 years, respectively), and functional assays are needed to ascertain the effect of the mutation.

The application of an easy-to-use, flexible sequencing method for the diagnosis of hereditary iron overload and HF has become a subject of great interest. The precise diagnosis of these disorders is clinically useful, especially from a therapeutic point of view. Phlebotomies, the main treatment for iron overload in nonanemic patients, are a well-tolerated and effective way to treat HH, but they can lead to anemia in class 1 ferroportin disease (17) and are contraindicated in HHCS. The availability of the causative mutation is also useful for genetic counseling of family members. This information offers the possibility of early diagnosis in relatives and may help prevent severe complications, as in JH (36).

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References


33. Beutler E, Griffin MJ, Gelbart T, West C. A previously unde-


