Abnormal regulation of HFE mRNA expression may not contribute to primary iron overload

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ABSTRACT

Background and Objectives. Hereditary hemochromatosis (HHC) is a common, recessively inherited, genetic disorder associated with an abnormality of the HFE gene. Subjects homozygous for a point mutation in the gene coding sequence, leading to the amino acid substitution C282Y, are usually affected by the disease. A second point mutation, causing the amino acid substitution H63D, has been described, and compound heterozygotes for the two mutations or homozygotes for the H63D mutation are at risk of developing a milder form of HHC. In populations of northern European origin the C282Y substitution accounts for more than 90% of cases of HHC. In Italy, however, fewer than 70% of patients with HHC are homozygous or compound heterozygous for HFE mutations. Even in the absence of mutations in its coding region, the HFE gene might be involved in the pathogenesis of HHC through inhibition of transcription of the gene or reduced stability of its mRNA.

Design and Methods. Since little is known about the regulation of HFE expression, we investigated 17 subjects heterozygous for one of the HFE mutations and with biochemical evidence of iron overload and compared the levels of wild type and mutated mRNAs in their peripheral blood cells. c-DNA regions flanking the mutated codons were amplified by reverse transcriptase polymerase chain reaction (PCR). PCR products derived from the two alleles were differentiated and quantified by digestion with restriction enzymes, electrophoresis in an agarose gel stained with ethidium bromide and densitometric scanning of the gel.

Results. In all cases wild type and mutated mRNAs were expressed at similar levels, suggesting that reduced expression of an HFE allele coding a normal protein is not involved in the pathogenesis of iron overload. However, we can not rule out that a tissue specific regulation of HFE expression in the cells directly involved in iron absorption is altered and contributes to the pathogenesis of the disease.

Interpretation and Conclusions. Our results suggest that primary iron overload is a multigenic syndrome; this hypothesis is strongly supported by the recent demonstration that the juvenile hemochromatosis locus maps to human chromosome 1q.

Key words: hereditary hemochromatosis (HHC), HFE mRNA, point mutations, coding sequence, RT-PCR

Heredity hemochromatosis (HHC) is one of the most frequent human genetic diseases, with a prevalence of 1 in 500 to 1 in 200 among Caucasians. If not correctly diagnosed and treated, HHC can be fatal and cause a high degree of morbidity, mainly related to heart and liver involvement and to endocrine dysfunction. HFE is the candidate gene responsible for the disease; it is related to the major histocompatibility complex (MHC) class I family and is located on human chromosome 6p. The homozygous status for a point mutation in the gene coding sequence, causing the amino acid substitution C282Y, is found in more than 90% of affected subjects of northern European descent. In Italy, however, the C282Y mutation accounts for fewer than 70% of HHC cases. A second point mutation, causing the H63D amino acid substitution, has been described, but its role in the pathogenesis of iron overload is still questioned, although compound heterozygotes for the two mutations or homozygotes for the H63D mutation usually show some degree of iron overload. Several new mutations in the HFE gene have recently been described, but most of them are probably simple polymorphisms. Only two other point mutations, one causing the S65C amino acid substitution and one altering messenger RNA splicing, have been convincingly shown to cause iron overload when associated with the C282Y mutation. However, these new mutations are rare, S65C being found in only 7.8% of HHC chromosomes negative for the other mutation, and do not explain the high frequency of negative cases in the Italian population.

Findings in Italy and the recent description of new entities such as a dysmetabolic iron overload syndrome, a juvenile form of genetic hemochromatosis, and African iron overload suggest that primary iron overload syndromes are heterogeneous and different molecular lesions in the HFE gene or in other genes can be responsible for such conditions.
However, in some cases of HLA-linked HHC, negative for classic HFE mutations, sequencing of HFE c-DNA and its intron-exon boundaries did not reveal additional mutations. One possible explanation of iron overload with an abnormality of the HFE gene which does not involve its coding sequence is that loss of HFE function derives from inhibition of its expression. Although a promoter region for the HFE gene has been identified, no information is currently available on the regulation of HFE expression, the function of its promoter or on other regulatory sequences. We therefore investigated subjects with some biochemical evidence of iron overload, but heterozygous for one of the HFE mutations associated with HHC, and evaluated the relative expression of the two alleles using a RT-PCR procedure. If suppression of HFE transcription or instability of its mRNA were responsible for reduced HFE synthesis from the allele encoding a normal HFE protein, lower levels of the normal transcript, compared to transcripts from mutated alleles, would be found.

**Design and Methods**

We analyzed 17 subjects who were heterozygous for one of the HFE gene mutations involved in HHC. Data about the patients are reported in Table 1. Patients #1-4 had been diagnosed as having HHC, although no family studies were available; iron overload had been previously documented by means of liver biopsy, and treatment by venesection was instituted. In 6 patients a high transferrin saturation and/or serum ferritin were detected, and subsequently confirmed, during a work up for obesity (patient #5) and on occasional check up procedures (patients #6-8, 16 and 17). Patients #9-11 were blood donors in whom iron status evaluation showed signs of iron overload and heterozygous for the H63D mutation. A 1 µg sample of RNA was reverse transcribed in a final volume of 20 µL containing 100 U of Moloney murine leukemia virus reverse transcriptase in 1x reverse transcription buffer, random hexamers and 20 U RNasin inhibitor (all from Promega Biotech, Madison WI, USA), and dithiothreitol 0.01 mol/L. Reverse transcription was allowed to proceed for 60 minutes at 42°C, followed by inactivation of reverse transcriptase for 10 minutes at 95°C. Two 2.5 µL aliquots of the reverse transcription reaction underwent two rounds of amplification by PCR, using nested primers, in order to detect the H63D and C282Y mutations. A 1 µL sample of RNA was reverse transcribed in a final volume of 20 µL containing 100 U of Moloney murine leukemia virus reverse transcriptase in 1x reverse transcription buffer, random hexamers and 20 U RNasin inhibitor (all from Gibco BRL, Gaithersburg, MD, USA), 0.5 mmol/L of each deoxyribonucleotide triphosphate (Boehringer Mannheim, Germany), 0.2 mg/mL bovine serum albumin (Promega Biotech, Madison WI, USA), and dithiothreitol 0.01 mol/L. Reverse transcription was allowed to proceed for 60 minutes at 42°C, followed by inactivation of reverse transcriptase for 10 minutes at 95°C. Two 2.5 µL aliquots of the reverse transcription reaction underwent two rounds of amplification by PCR, using nested primers, in order to detect the H63D and C282Y mutations. The template used in the second round amplification was 1 µL of the first round PCR product. Amplification conditions were essentially as previously described, except for primers. RT-PCR primer sequences are reported in Table 2 and were derived from the HFE c-DNA sequence as determined by Feder et al. and deposited in GenBank (U60319). Second round PCR products were digested with restriction endonucleases and analyzed as described for DNA mutation screening. Codon 63 analysis yields a 272 bp amplification product with three Mbo I restriction fragments of 118, 99 and 55 bp for the wild type allele and two fragments, 217 and 55 bp in size, for the mutated allele. Therefore, a heterozygous sample shows four bands. At codon 282 the undigested product is 347 bp long with Rsa I restriction fragments of 209 and 138 bp for the wild type and

### Table 1. **Phenotype and genotype of the subjects investigated in the present study.**

<table>
<thead>
<tr>
<th>Pt</th>
<th>Sex</th>
<th>Age (years)</th>
<th>Serum iron (µg/dL)</th>
<th>Transferrin saturation (%)</th>
<th>Serum ferritin (ng/mL)</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>M</td>
<td>56</td>
<td>194</td>
<td>73</td>
<td>983</td>
<td>+/- -/-</td>
</tr>
<tr>
<td>2</td>
<td>M</td>
<td>47</td>
<td>225</td>
<td>83</td>
<td>672</td>
<td>+/- -/-</td>
</tr>
<tr>
<td>3</td>
<td>M</td>
<td>71</td>
<td>117</td>
<td>41</td>
<td>650</td>
<td>+/- -/-</td>
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<tr>
<td>4</td>
<td>M</td>
<td>64</td>
<td>146</td>
<td>65</td>
<td>1,214</td>
<td>+/- -/-</td>
</tr>
<tr>
<td>5</td>
<td>M</td>
<td>17</td>
<td>152</td>
<td>50</td>
<td>61</td>
<td>+/- -/-</td>
</tr>
<tr>
<td>6</td>
<td>M</td>
<td>26</td>
<td>280</td>
<td>86</td>
<td>79</td>
<td>+/- -/-</td>
</tr>
<tr>
<td>7</td>
<td>F</td>
<td>56</td>
<td>108</td>
<td>70</td>
<td>1,310</td>
<td>+/- -/-</td>
</tr>
<tr>
<td>8</td>
<td>M</td>
<td>58</td>
<td>123</td>
<td>48</td>
<td>747</td>
<td>+/- -/-</td>
</tr>
<tr>
<td>9</td>
<td>M</td>
<td>45</td>
<td>130</td>
<td>37</td>
<td>400</td>
<td>+/- -/-</td>
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<tr>
<td>10</td>
<td>F</td>
<td>51</td>
<td>104</td>
<td>33</td>
<td>501</td>
<td>+/- -/-</td>
</tr>
<tr>
<td>11</td>
<td>M</td>
<td>49</td>
<td>90</td>
<td>33</td>
<td>455</td>
<td>+/- -/-</td>
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<tr>
<td>12</td>
<td>M</td>
<td>37</td>
<td>242</td>
<td>95</td>
<td>616</td>
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</tr>
<tr>
<td>13</td>
<td>M</td>
<td>65</td>
<td>219</td>
<td>86</td>
<td>828</td>
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<tr>
<td>14</td>
<td>M</td>
<td>36</td>
<td>169</td>
<td>65</td>
<td>454</td>
<td>+/- -/-</td>
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<tr>
<td>15</td>
<td>M</td>
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<td>211</td>
<td>87</td>
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<tr>
<td>16</td>
<td>M</td>
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<td>59</td>
<td>431</td>
<td>+/- -/-</td>
</tr>
<tr>
<td>17</td>
<td>F</td>
<td>29</td>
<td>151</td>
<td>48</td>
<td>53</td>
<td>+/- -/-</td>
</tr>
</tbody>
</table>
| *+* and – indicate the presence and the absence of the mutation, respectively. All patients were negative for the S65C mutation; M=male; F=female.

For evaluation of the relative expression of mutated and wild type HFE alleles, total RNA was isolated by the guanidium-thiocyanate method except for the peripheral blood mononuclear cells of subjects with signs of iron overload and heterozygous for the H63D or the C282Y mutations. A 1 µL sample of RNA was reverse transcribed in a final volume of 20 µL containing 100 U of Moloney murine leukemia virus reverse transcriptase in 1x reverse transcription buffer, random hexamers and 20U RNasin inhibitor (all from Gibco BRL, Gaithersburg, MD, USA), 0.5 mmol/L of each deoxyribonucleotide triphosphate (Boehringer Mannheim, Germany), 0.2 mg/mL bovine serum albumin (Promega Biotech, Madison WI, USA), and dithiothreitol 0.01 mol/L. Reverse transcription was allowed to proceed for 60 minutes at 42°C, followed by inactivation of reverse transcriptase for 10 minutes at 95°C. Two 2.5 µL aliquots of the reverse transcription reaction underwent two rounds of amplification by PCR, using nested primers, in order to detect the H63D and C282Y mutations. The template used in the second round amplification was 1 µL of the first round PCR product. Amplification conditions were essentially as previously described, except for primers. RT-PCR primer sequences are reported in Table 2 and were derived from the HFE c-DNA sequence as determined by Feder et al. and deposited in GenBank (U60319). Second round PCR products were digested with restriction endonucleases and analyzed as described for DNA mutation screening. Codon 63 analysis yields a 272 bp amplification product with three Mbo I restriction fragments of 118, 99 and 55 bp for the wild type allele and two fragments, 217 and 55 bp in size, for the mutated allele. Therefore, a heterozygous sample shows four bands. At codon 282 the undigested product is 347 bp long with Rsa I restriction fragments of 209 and 138 bp for the wild type and
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209, 109 and 29 bp for the mutated alleles, respectively. Since the 29 bp fragment is not visible on the gel, a heterozygous sample shows three bands.

A Kodak Digital Science Electrophoresis Documentation and Analysis System was used for gel imaging and band densitometry. The procedure for evaluation of the relative expression of two HFE alleles was validated by performing multiple dilutions of RNA samples carrying a mutation with wild type samples and determining the ratios between bands derived from different alleles.

Results

While analyzing subjects who had biochemical evidence of iron overload, we found 17 cases heterozygous for one of the HFE mutations associated with iron overload. We, therefore, amplified HFE cDNA from these patients in order to evaluate the relative expression of the mutated and the apparently wild type alleles. Preliminary experiments, in which RNA from subjects homozygous for one mutation was diluted with RNA from normal subjects, showed that the ratio between bands derived from different alleles, evaluated by densitometric analysis, changed according to dilution (Figure 1). This indicates that the two alleles, which differ for a point mutation, are amplified with the same efficiency, and RT-PCR is a reliable method for evaluating their relative expression. Lower ratios between samples were not tested to assess the limit of sensitivity of the method since our aim was to detect significant differences between alleles, not to detect very low levels of expression of one allele compared to another. In addition, since primers were chosen in order to produce bands similar in size to those obtained from DNA amplification, a similar ratio between bands in PCR products from undiluted DNA and RNA of heterozygous subjects would indicate similar expression of the two alleles. No sample had the S65C mutation.

Figure 2 shows representative amplifications from both DNA and RNA. All of the samples behaved similarly to the ones in Figure 2, and densitometric scanning confirmed the similar expression levels for the wild type and the mutated alleles. The observed pat-
Discussion

Since HHc is inherited as an autosomal recessive disease, it is still uncertain whether subjects with some degree of iron overload and heterozygous for one of the HFE gene mutations involved in HHC, in the absence of other known reasons for having increased transferrin saturation and/or serum ferritin levels, really have an HFE-related disorder of iron metabolism. While analyzing the HFE genotype of subjects showing biochemical evidence of iron overload, we found 14 patients who were heterozygous for the H63D mutation and 3 patients heterozygous for the C282Y mutation of the HFE gene (however, one C282Y heterozygous patient had an iron loading anemia). None of these subjects was compound heterozygous for the two mutations or had the recently described S65C mutation. It is usually assumed that the phenotypic expression of gene lesions involving loss of function occurs when both copies of the gene are damaged. Since other mutations in the HFE gene c-DNA either represent polymorphisms or are extremely rare, we must consider the possibility that reduced transcription or instability of the mRNA encoding the wild type protein contributes to iron overload. Given that the two alleles differ for a single point mutation, c-DNA regions containing the mutation in heterozygous subjects are supposed to be amplified with the same efficiency by RT-PCR, which should, therefore, be a reliable method for evaluating the relative expression of different HFE alleles in peripheral blood cells. Functional characterization of the recently described HFE promoter may shed some light on this possibility.

Potential implications for clinical practice

♦ Our results suggest that primary iron overload syndromes may not be due to abnormal expression of the HFE gene. Abnormal function of different genes may contribute to primary iron overload, as indicated by the recent demonstration that the juvenile hemochromatosis locus maps to human chromosome 1q.
References