Digenic inheritance of mutations in HAMP and HFE results in different types of haemochromatosis

Alison T. Merryweather-Clarke1,*, Estelle Cadet2, Adrian Bomford3, Dominique Capron4, Vip Viprakasit1,5, Anne Miller6, Paddy J. McHugh7, Roger W. Chapman8, Jennifer J. Pointon1, Victoria L.C. Wimhurst1, Karen J. Livesey1, Voravarn Tanphaichitr5, Jacques Rochette2 and Kathryn J.H. Robson1

1MRC Molecular Haematology Unit, Weatherall Institute of Molecular Medicine, Headley Way, Oxford OX3 9DS, UK, 2Génétique Médicale-CHU, Faculté de Médecine, Université Jules Verne de Picardie, Amiens, France, 3Institute of Liver Studies, Kings College Hospital, London, UK, 4Hépato-gastro-entérologie, Centre Hospitalier Universitaire, Amiens, France, 5Department of Paediatrics, Faculty of Medicine, Siriraj Hospital, Mahidol University, Bangkok, Thailand, 6Department of Pathology, St Peter’s Hospital, Chertsey, UK, 7Department of Pathology, Kingston Hospital, Kingston, UK and 8Department of Gastroenterology, John Radcliffe Hospital, Headley Way, Oxford, OX3 9DU, UK

Received May 30, 2003; Revised June 26, 2003; Accepted July 5, 2003

Haemochromatosis (HH) is a clinically and genetically heterogeneous disease caused by inappropriate iron absorption. Most HH patients are homozygous for the C282Y mutation in the HFE gene. However, penetrance of the C282Y mutation is incomplete, and other genetic factors may well affect the HH phenotype. Ferroportin and TFR2 mutations also cause HH, and two HAMP mutations have recently been reported that causes juvenile haemochromatosis (JH) in the homozygous state. Here, we report evidence for digenic inheritance of HH. We have detected two new HAMP mutations in two different families, in which there is concordance between severity of iron overload and heterozygosity for HAMP mutations when present with the HFE C282Y mutation. In family A, the proband has a JH phenotype and is heterozygous for C282Y and a novel HAMP mutation Met50del IVS2+1(-G). This is a four nucleotide ATGG deletion which causes a frameshift. The proband’s unaffected mother is also heterozygous for Met50del IVS2+1(-G), but lacks the C282Y mutation and is heterozygous for the HFE H63D mutation. Met50del IVS2+1(-G) was absent from 642 control chromosomes. In family B, a second novel, less severe HAMP mutation, G71D, was identified. This was detected in the general population at an allele frequency of 0.3%. We propose that the phenotype of C282Y heterozygotes and homozygotes may be modified by heterozygosity for mutations which disrupt the function of hepcidin in iron homeostasis, with the severity of iron overload corresponding to the severity of the HAMP mutation.

INTRODUCTION

Hereditary haemochromatosis (HH) is a disease caused by inappropriate dietary iron absorption (1), and is one of the most common autosomal recessive diseases affecting populations of north European origin. Those affected by HH continue to absorb iron despite excess iron stores, resulting in accumulation of iron in various tissues including the liver, heart and pancreas, which may cause fatal organ damage during middle age or thereafter. However, if excess iron stores are reduced by phlebotomy before the development of organ failure, life expectancy is normal (2). Over 80% of north European HH patients are homozygous for the C282Y mutation in the HH gene HFE (chromosome 6 band p22.1), and a high proportion of the remaining patients are compound heterozygotes for C282Y and the common HFE mutation H63D (3). Depending on the population studied, 4–35% of cases presenting with the HH phenotype carry only a single C282Y or H63D allele or none at all (4). HH is a heterogeneous disease with penetrance in C282Y homozygotes varying considerably, which has discouraged widespread

*To whom correspondence should be addressed. Tel: +44 1865222388; Fax: +44 1865222500; Email: alison@hammer.imm.ox.ac.uk
Genetic screening of at-risk populations. The results of a number of population studies comparing HFE genotype with serum iron parameters suggest that transferrin saturation (Tsat) correlates with HFE genotype but that serum ferritin concentration (sF) does not (5–7). The number of C282Y homozygotes identified through population genotyping is in many cases at least an order of magnitude greater than the number of HH patients (5,8,9). This has given rise to active debate regarding penetrance and HH (10,11). Studies comparing the degree of iron overload in mice lacking Hfe provide further evidence to suggest that other genetic factors contribute to iron overload (12,13). These data support the hypothesis that HH should no longer be considered a monogenic disease but rather an oligogenic disorder, where homozygosity for the C282Y mutation predisposes an individual to a raised transferrin saturation (Tsat) but is insufficient to result in HH (5–7).

Mutations in the transferrin receptor 2 gene TFR2 (chromosome 7, band q22) (14,15) and in ferroportin I (SLC11A3, chromosome 2, band q32) (16,17) have been reported to cause HH, and juvenile haemochromatosis (JH) patients have been described who are homozygous for mutations in the hepcidin gene HAMP (18) on chromosome 19, band q13. Hepcidin is a recently identified antimicrobial peptide synthesized by hepatocytes in response to bacterial infection and inflammation (19,20). Recent studies have implicated HFE in regulation of HAMP expression (21,22).

With the aim of identifying other mutations or polymorphisms that might contribute to the variable penetrance seen in HH, we have identified two new HAMFP mutations in two different families who have combinations of mutations in both HFE and HAMP.

RESULTS

In family A (Table 1 and Fig. 1), the proband (IIv) presented at age 27 years with congestive heart failure, general fatigue, hypogonadotrophic hypogonadism, type 1 diabetes, skin pigmentation and hepatomegaly. Fibrosis was detected following liver biopsy, when the hepatic iron concentration was 481 μmol g−1 dry liver (normal <36 μmol g−1) with a hepatic iron index of 17.8 (normal <1.9), serum ferritin (sF) 1645 μg l−1 (normal 15–300 μg l−1), and Tsat 92% (normal 17–45%). Removal of 27 g of iron over 4 years restored the Tsat to 30% and sF to 40 μg l−1. Tsat was found to be >80% following each of several periods of inattendance for maintenance phlebotomy, with sF remaining below 200 μg l−1. Unless at least 0.5 g of iron is removed per month, the Tsat goes up to >80% in about 3 months. A total of about 80 g iron has been removed during 19 years of inconsistent attendance for phlebotomy. These data are characteristic of a severe form of iron loading such as is found in patients with JH.

HFE genotyping identified the proband (Iv) as an HFE C282Y heterozygote. Denaturing high-performance liquid chromatography (dHPLC) and sequence analysis of HAMP exons 2 and 3 from Iv revealed heterozygosity for a four nucleotide ATGG deletion Met50del IVS2+1(-G) which removes the last three nucleotides of exon 2 and the first one of intron 2. This deletion retains the splice site consensus sequence but alters the reading frame, which then extends beyond the end of the normal transcript. The proband’s mother (Iii) was heterozygous for the same HAMP frameshift mutation and the HFE mutation H63D. Other family members lacked the HAMP mutation but had mutations in HFE (Fig. 1). The proband’s mother (Iii) is aged 86 years with no major clinical problems and has normal haematological and biochemical parameters (Table 1). The proband’s surviving brother (IIIi) is an HFE H63D homozygote with normal iron indices (Table 1).

Three other siblings all died prematurely, two brothers (Ii and Iii) from primary pancreatic cancer aged 51 years, and one sister (IIIi) from a cerebral aneurysm aged 55 years.

The proband in family B (Iii) was diagnosed with haemochromatosis aged 35 (Table 1 and Fig. 2) and was

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**Table 1.** Biochemical parameters of family members and a control with HAMP mutations

<table>
<thead>
<tr>
<th>Family A</th>
<th>Family B</th>
<th>Control sample</th>
<th>Normal values</th>
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<tr>
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<tr>
<td>Age when</td>
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<td></td>
</tr>
<tr>
<td>tested (years)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Iii</td>
<td>86</td>
<td>60</td>
<td>27</td>
</tr>
<tr>
<td>IIIi</td>
<td>86</td>
<td>60</td>
<td>27</td>
</tr>
<tr>
<td>IIv</td>
<td>35</td>
<td>35</td>
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</tr>
<tr>
<td>Gender</td>
<td>F</td>
<td>M</td>
<td>M</td>
</tr>
<tr>
<td>Tsat (%)</td>
<td>35</td>
<td>35</td>
<td>92</td>
</tr>
<tr>
<td>Ferritin (μg/l)</td>
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<td>287</td>
<td>1645</td>
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<tr>
<td>Iron (μmol/l)</td>
<td>16</td>
<td>18.3</td>
<td>42</td>
</tr>
<tr>
<td>γGT* (iu/l)</td>
<td>14</td>
<td>24</td>
<td>56</td>
</tr>
<tr>
<td>ALT* (iu/l)</td>
<td>16</td>
<td>34</td>
<td>62</td>
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<tr>
<td>RBC (×10^6/mm³)</td>
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<td>5.3</td>
<td>5.24</td>
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<tr>
<td>Hb (g/l)</td>
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<td>MCV* (fl)</td>
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<td>100</td>
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<tr>
<td>Platelets (×10^9/l)</td>
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<td>256</td>
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<tr>
<td>Glucose (mmol/l)</td>
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<td>5.83</td>
<td>10</td>
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<tr>
<td>HFE* genotype</td>
<td>HD/CC</td>
<td>DD/CC</td>
<td>HH/CY</td>
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<tr>
<td>HAMP genotype</td>
<td>Met50del IVS2+1(G) +/−</td>
<td>WT Met50del IVS2+1(G) +/−</td>
<td>WT</td>
</tr>
</tbody>
</table>

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*γGT, γ-glutamyl transferase; ALT, alanine amino transferase; Hb, haemoglobin; MCV, mean corpuscular volume; WT, wild-type.
found to be a C282Y homozygote. Liver function and ultrasound results were normal, apart from a raised alanine amino transferase (ALT). Subsequent removal of 6 g iron by phlebotomy over 8 months restored the sF to normal, and maintenance therapy is four units annually. Subsequent analysis showed that his father (Ii) was a C282Y heterozygote, his sister (IIi) was a C282Y homozygote, and both had elevated sF (Table 1). The proband’s sister (IIi) also had a raised sF and non-inflammatory arthritis, mainly in her metacarpophalangeal joints. Subsequent removal of 3.5 g iron over 12 months by phlebotomy restored her sF to normal levels, and she is now starting a course of maintenance venesection. Mutation detection and sequence analysis identified a missense mutation in HAMP, the substitution of glycine 71 by aspartic acid (G71D) due to a G→A substitution in exon 3 of the HAMP gene (212 G→A). The proband’s father (Ii) and sister (IIi) are both heterozygous for this mutation (Fig. 2). Biochemical and haematological parameters for this family are given in Table 1.

The HAMP Met50del IVS2+1(-G) mutation was absent from 642 control chromosomes. Three G71D heterozygotes were found in 1022 control chromosomes, giving an allele frequency of 0.3%. Biochemical data was available for one of these G71D heterozygotes; parameters were in the normal range (Table 1).

**DISCUSSION**

The HFE protein is an atypical MHC class I protein that has been shown to bind to transferrin receptor I and thus modify its affinity for apotransferrin (23,24). HFE is expressed in placenta, crypt cells of the duodenum, Kupffer cells and macrophages (25–27). The C282Y mutation abrogates the formation of the disulfide bond in the α3 domain of HFE, thus
preventing assembly with β2-microglobulin (28,29). Mutant protein collects in the Golgi and is rapidly degraded, thus it fails to reach the cell surface efficiently (29). The H63D mutation in HFE occurs in the α1 domain; this protein is correctly processed and is found on the cell surface (29). The H63D mutation is very much milder than the C282Y mutation.

Hepcidin is a recently identified antimicrobial peptide synthesized in the liver (19,20) as an 84 amino acid prepropeptide, which is processed to give an active peptide comprising the 25 amino acid C-terminal portion of the molecule (19). Nuclear magnetic resonance spectrometry suggests that the active portion of the molecule is a simple hairpin held together by a number of disulfide bridges in a ladder-like configuration (30). The positively charged hydrophilic side chains are spatially separated from those that are hydrophobic, a common feature of peptides that disrupt bacterial membranes. The Met50del IVS2+1(-G) frameshift deletion occurs at the end of exon 2, completely disrupting expression of the active peptide which is encoded by exon 3. Hence no functional hepcidin will be produced from the mutant allele. The G71D mutation changes the charge of amino acid 71, which is between the third and fourth cysteines, residues 70 and 72 (Fig. 2D). This neutral to acidic change, at the end of the first β pleated sheet of the molecule, is likely to be functionally significant. The only other amino acid with an acidic side chain is at position 60, the start of the processed hepcidin molecule. In pig, rodent and fish hepcidins, amino acid 71 is glycine, lysine or asparagine, respectively. Hence we predict that the G71D mutation is likely to affect the activity of hepcidin. Mice lacking functional Hamp genes develop spontaneous iron overload (31), as do those lacking functional Hfe and β2-microglobulin (32,33). There is no evidence that hepcidin interacts directly with HFE.

Recently two families have been described where JH segregates with either homozygosity for a frameshift or nonsense mutation in HAMP (18). The heterozygotes in these
families are reported to have a normal phenotype. The Met50del IVS2+1(-G) mutation in family A results in a similar disordered protein to that described by Roetto et al. (18). In both untreated HH patients who are C282Y homozygotes and Hfe knockout mice, hepcidin levels are significantly decreased despite the increased iron loading (34). Together these data suggest that HFE may play a role in regulating hepcidin in response to iron loading. Hence, in individuals who carry both HFE and hepcidin mutations, reduced HFE function may act synergistically with reduced hepcidin levels, resulting in iron overload.

In family A, a clear synergistic effect is observed between the HAMP Met50del IVS2+1(-G) mutation and the HFE C282Y, but not the H63D, mutation, suggesting that the failure of HFE to reach the cell surface is important in regulating hepcidin expression levels. This is consistent with two recent reports; hepcidin deficiency caused by Hfe deficiency seems to be the primary pathogenic factor mediating iron overload in Hfe-related haemochromatosis in mice (21), and constitutive hepcidin expression has recently been reported to prevent iron overload in a mouse model of haemochromatosis (22). The iron loading seen in the proband in family A is severe and of early onset, which correlates with the fact that homozygosity for a similar frameshift mutation against a wild-type HFE background also leads to JH (18). The phenotype in family B is much milder, the double heterozygote having been identified indirectly through family screening. Hepcidin carrying the G71D mutation is likely to have reduced activity, and in conjunction with homozygosity for C282Y leads to less severe disease than that of patients who have the hepcidin mutation Met50del IVS2+1(-G). The frequency of the Met50del IVS2+1(-G) and G71D mutations differ, the former being rare but G71D being relatively common.

All three individuals with HFE C282Y and HAMP mutations had an increased mean corpuscular volume (MCV) at presentation (Table 1). There is no obvious explanation for the raised MCV in IIV and IIII in families A and B respectively; factors such as alcohol consumption, thyroid function, increased reticulocyte counts, vitamin B12 and folate deficiency have been excluded. While there was an unexplained macrocytosis in III, family B, who is a hepcidin G71D heterozygote, her brother (IIii), also an HFE C282Y homozygote but with a wild-type HAMP gene, had a normal MCV. Alcohol consumption cannot be excluded as a cause of macrocytosis for their father. MCV is also increased in phenylhydrazine-treated mice, whose liver hepcidin mRNA concentration is reduced by a factor of 2, total liver iron is increased by a factor of 4 and serum iron by a factor of 3 (35), consistent with our observations.

Our data suggest that mechanisms including digenic inheritance as seen in severe insulin resistance (36) and/or of triallelic inheritance similar to that observed in Bardet–Biedl syndrome (37) may be operating in haemochromatosis (38). This would explain the heterogeneity of the haemochromatosis phenotype, in which homozygosity for the HFE C282Y mutation is necessary but not sufficient in many cases for development of the disease phenotype. The variation in iron overload seen in Hfe–/– mice with different genetic backgrounds (12,13) could also be explained by such mechanisms. The natural mutations and phenotypes observed in our patients are proof of predictions made from mouse models of haemochromatosis that genetic variation in HAMP might contribute to the wide range in phenotypic variation in HFE C282Y homozygotes (21,22). We propose that the phenotype of C282Y heterozygotes and homozygotes may be modified by heterozygosity for mutations which disrupt the function of hepcidin in iron homeostasis, with the severity of iron overload corresponding to the severity of the HAMP mutation. Our data also suggest that heterozygosity for the as yet unidentified JH gene mapping to 1q21 may contribute, when present in conjunction with the HFE C282Y allele, to the burden of iron overload in Italy (39).

**MATERIALS AND METHODS**

**Subjects and consent**

Informed consent was obtained from all participating individuals and the study was approved by the respective Ethical Committees in the UK, France and Thailand. Family members, patients and anonymized controls were of north European origin.

**Analysis of HAMP**

Amplimers were designed to incorporate all known coding sequences and splice sites (Genbank AD000684). A 262 bp fragment containing HAMP exon 1 was amplified using sense primer 5’ AGCAAAAGGGAGGCGCTACAGACCAC and antisense primer 5’ TCCCATCCCTGCTGCCTCTATAGGAC. A 499 bp fragment containing HAMP exons 2 and 3 was amplified using sense primer 5’ TTGCCGGAGCAGCTCTCAGAGGTTCCAC and antisense primer 5’ TGCAAGGCGA-GGTCAGGACAAGCTCTTATAGC. DHLPC analysis of exon 1 proceeded at 64°C, and of exons 2 and 3 at 61.5 and 62.7°C according to the manufacturer’s specifications (Transgenomic). All anomalous traces were subjected to automated sequencing on a 3100 capillary sequencer (Applied Biosystems). Where possible, restriction fragment length polymorphism (RFLP) was used to confirm sequencing results. The Met50del IVS2+1(-G) mutation destroys a BstFI site and the G71D mutation is likely to have reduced activity, and in conjunction with homozygosity for C282Y leads to less severe disease than that of patients who have the hepcidin mutation Met50del IVS2+1(-G). The frequency of the Met50del IVS2+1(-G) and G71D mutations differ, the former being rare but G71D being relatively common.

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Analysis of HFE and SLC11A3

Affected family members were screened for novel mutations in ferroportin (SLC11A3) and HFE and found to be negative. Members of family A were negative for the Y250X mutation in transferrin receptor 2.

ACKNOWLEDGEMENTS

We are grateful to individuals, families and members of the Haemochromatosis Society who participated in this research. We thank N. Elanko, I. Taylor and K. Clark for technical assistance, and Professor Sir David Weatherall and Christine Patch for helpful discussion. This work was funded by EC contract QLRT-1999-02237.

REFERENCES


