The recently identified type 2A juvenile haemochromatosis gene (HJV), a second candidate modifier of the C282Y homozygous phenotype

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The most common form of hereditary haemochromatosis is an adult-onset condition usually associated with the HFE C282Y/C282Y genotype. The phenotypic expression of this genotype is heterogeneous and depends on a complex interplay of genetic and non-genetic factors. The aim of the present study was to determine if mutations in the recently identified HJV gene were associated with more severe iron overload phenotypes in C282Y homozygous patients. From a cohort of 310 C282Y homozygous patients, we found nine (six males and three females) with an additional HJV missense mutation in the heterozygous state (S105L, E302K, N372D, R335Q or the previously described L101P and G320V). The iron indices of eight patients appeared to be more severe than those observed in C282Y homozygous patients of identical sex and similar age ranges. The mean serum ferritin concentration of the six males with an HJV mutation was significantly higher than that of C282Y homozygous males without an additional mutation [2350.3 (s = 1429.9) versus 1227.2 (s = 1130.1) µg/l; P = 0.0233, Student’s t-test]. We have recently reported that mutations in the gene that encodes hepcidin (HAMP) could explain one part of the C282Y/C282Y-related phenotypic heterogeneity by accentuating the iron burden. Our new data reveal that mutations in the HJV gene could be associated with a similar effect. Taken together, these results emphasize that a search for modifier genes could enable us to more precisely distinguish those C282Y homozygous patients with a higher risk to develop a severe iron overload and, consequently, clinical complications.

INTRODUCTION

Hereditary haemochromatosis (HH) now refers to six inherited disorders of iron metabolism characterized by progressive iron accumulation in major organs (1). If not recognized and treated, this progressive iron loading can lead to tissue damage and severe clinical complications, including cirrhosis, hepatocellular carcinoma and cardiomyopathy (2). The historical and most prevalent form of HH, called HH type I, is an adult-onset autosomal recessive condition caused by mutations in HFE (OMIM 235200) (1,3). Less common forms of HH are principally distinguished by their transmission pattern (autosomal recessive or autosomal dominant), by the course of disease (adult or juvenile onset) and by the predominant localization of hepatic iron deposition (hepatocytes or Kupffer cells). They include two early onset autosomal recessive disorders, called juvenile haemochromatosis type 2A and 2B (OMIM 602390), respectively, caused by mutations in HJV (OMIM 608374) that encodes hemojuvelin (4), and in HAMP (OMIM 606464) that encodes hepcidin (5).

The HJV gene has been recently implicated in the most frequent form of juvenile haemochromatosis on the basis of the description of six mutations, found in either a homozygous or compound heterozygous state, in 12 unrelated families of Greek, Canadian or French descent (4). From this precept result, 17 other mutations have been reported in 31 families from Southeast USA, Italy, Saguay-Lac-Saint Jean (Quebec), England, Albania, Australia and France (6,7). One mutation, the G320V amino acid change, appears to be common as it has been detected in 34 of 60 (56.7%) juvenile haemochromatosis patients from five different populations. The 22 other mutations have been mainly reported in single families, thus highlighting an important allele heterogeneity.
Between 64 and 100% HH patients of European origin are homozygous for the C282Y HFE gene mutation (8). The phenotypic expression of this genotype is heterogeneous and could be modulated by environmental factors, modifier genes and epigenetic factors (1,9). In accordance with the demonstration by Nicolas et al. (10) of an increased hepatic iron loading in Hfe \(-/-\) mice with an additional HAMP deficient allele, we have previously proposed that heterozygous HAMP mutations could explain one part of the C282Y/C282Y-related phenotypic heterogeneity by accentuating the iron burden (11).

The aim of the present study was to determine if mutations in the HJV gene could be associated with a similar effect. DHPLC scanning of the whole HJV coding region detected six heterozygous nucleic acid changes in nine of 310 C282Y homozygous patients. Two DNA variations resulted in the G320V and L101P amino acid changes, and have been described previously. The other four were found to correspond to four novel missense mutations (S105L, E302K, R335Q and N372D). On the basis of clinical manifestations and iron indices comparisons, we argue that iron overload phenotypes of eight of the nine C282Y homozygous patients with an additional HJV heterozygous mutation are amongst the most elevated. These data led us to conclude that the HJV gene also contributes to the severity of the main adult-form of HH.

RESULTS

DHPLC scanning of HJV in 310 C282Y homozygous patients and 333 control subjects

The present study included 310 C282Y homozygous patients and 333 control subjects. Five of the 310 C282Y homozygous patients were already known to be carriers of a heterozygous HAMP mutation. They did not display an additional HJV mutation.

In nine patients, DHPLC scanning of the whole HJV coding region revealed heterozygous nucleic acid changes in either exon 3 or 4. Subsequent sequencing analysis identified a cytosine to thymine transition at position 314, which changes amino acid 105 from serine to leucine (S105L) in one female and a guanine to adenine transition at position 904, which changes amino acid 302 from glutamic acid to lysine (E302K), in two non-related males; another guanine to adenine transition at position 1004, which changes amino acid 335 from arginine to glutamine (R335Q) in two siblings of opposite sexes; an adenine to guanine transition at position 1114, which changes amino acid 372 from asparagine to aspartic acid (N372D) in one female and the two previously described G320V and L101P mutations in two non-related males. DHPLC affected profiles and related sequencing electrophoregrams are shown in Figure 1.

Two of the observed mutations, L101P and E302K, were also detected in the heterozygous state in three of the 333 control subjects (allelic frequencies of two in 666 and one in 666, respectively). These three non-iron overload individuals were negative for HFE mutations.

The complete list of mutations is summarized in Table 1. Sexes and clinical manifestations observed in the HJV mutation carriers are also shown in this table.

Iron overload phenotypes of the nine C282Y homozygous patients with an additional mutation in the HJV gene

The two males with the known L101P mutation (males 1 and 2) exhibited a severe biological iron overload marked by serum ferritin levels ≥2000 μg/l before the third decade of life (Table 2). Arthropathy, skin pigmentation and cardiac failure were further observed in the patient with the more severe iron overload (Table 1), in whom 22.8 g of iron were removed by phlebotomies before he reached normal iron indices. The 50-year-old C282Y homozygous female who carried the common G320V mutation (female 2) displayed a transferrin saturation level of 93% and a serum ferritin concentration of 1232 μg/l (Table 3). Clinical manifestations included arthropathy and hepatic fibrosis (with a histological hepatic iron index of 0.74), and 18.1 g of iron had been removed by phlebotomy (Table 1).

When the three female and six male carriers of an additional HJV mutation were considered together, it appeared that their iron overload biological phenotypes were more severe than those observed in C282Y homozygous patients of identical sex and similar age ranges (Tables 2 and 3). This was particularly evident for the serum ferritin concentrations, and, using a logarithmic transformation in order to normalize the values, we found that the mean serum ferritin level of the six males with a HJV mutation was significantly different from that of the C282Y homozygous males (age range at diagnosis, 15–75 years) without an HJV or HAMP mutation \([2350.3 (\sigma = 1429.9) \text{versus} 1227.2 (\sigma = 1130.1) \text{μg/l}, P = 0.0233, \text{Student’s} \ t\text{-test}]\).

DISCUSSION

Most patients with HH are homozygous for the C282Y HFE mutation (8). At diagnosis, all of these patients display a biological phenotype characterized by elevated transferrin saturation levels with or without a serum ferritin increase. A significant percentage also display a therapeutic phenotype, retrospectively defined by the quantity of iron removed by phlebotomies to reach normal iron indices (5 g in men and 3 g in women), but only a small number display a clinical phenotype. The current perception of the disease conflicts with an earlier finding, which was biased by selection of patients with morbid complications, that the C282Y/C282Y genotype was highly penetrant. Even when considering biological phenotypic features and elderly individuals, population screenings and family studies have now confirmed that the main HH-associated genotype is not fully penetrant and that its phenotypic expression is very heterogenous (1,9,12–14).

As recognized by several authors, the pronounced variation in expression of the C282Y homozygous genotype probably depends on a complex interplay of genetic and non-genetic factors. However, if the influence of environmental components, such as excessive alcohol consumption or physiological and voluntary blood loss, has been well documented (1,15), the influence of epigenetic mechanisms or those of modifier genes is less obvious. Thus, despite clear indications that several genes of iron metabolism act as modifiers of the phenotypic expression in Hfe knockout mice (16–18), the association
between mutations or polymorphisms out of the \textit{HFE} gene and an increased iron loading in C282Y homozygous patients has only come from two \textit{HAMP} gene studies (11,19).

To address the question of whether \textit{HJV} could be a second modifier gene in HH type I, we studied the whole \textit{HJV} coding region in C282Y homozygous patients with mild to severe iron overload. In three of the patients, we detected the L101P and G320V missense mutations, which have been unequivocally implicated in the juvenile haemochromatosis type 2A form (4,6,7). We noted that the two males who carried the L101P mutation were included in a blood iron removing protocol at 23 and 30 years old, and that all three patient carriers of the additional known \textit{HJV} mutations displayed very important biological iron overload phenotypes and clinical manifestations.

Four novel missense mutations were found in six other patients. When considering the frequency of subjects carrying an \textit{HJV} mutation in the control population (3/333; 0.9%), the number of C282Y homozygous patients with an \textit{HJV} mutation was expected to be about three (cohort of 310 patients). We detected a total of nine. This is therefore a second argument that an additional \textit{HJV} mutation could confer an increased risk of displaying an iron overload.

To date, little is known on the hemojuvelin structure and function. We speculate that two of the four novel substitutions reported in our study occur in important regions of the HJV protein. Serine 105 is highly conserved between species. Its change to a leucine takes place within a cluster (including the S85P, G99V, G99R, and L101P previously described mutations) around the relevant RGD motif (6). Glutamic acid 302 is not conserved in zebrafish, in which it is replaced by an arginine, but it is included in a von Willebrand-like domain (residues 167–310). Therefore, its change to a leucine could have functional consequences. For the other two mutations, not observed on the 666 control chromosomes, we highlight that the N372D substitution leads to the change of a highly conserved amino acid, whereas the R335Q amino

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figures.jpg}
\caption{DHPLC affected elution profiles (in black) versus wild-type elution profiles (in green) for the L101P, S105L, E302K, G320V, R335Q and N372D \textit{HJV} missense mutations. Sequencing electrophoregrams are shown for both the wild-type and the mutated exon 3 or 4 related forward sequences (position of the mutation is indicated by arrow).}
\end{figure}
acid change takes place in a position not represented in zebrafish. This last missense mutation has been observed in two iron-loaded sibs. The brother (male 5) displayed a severe iron overload, as more particularly evidenced by a serum ferritin level of 2657 \( \mu \text{g/l} \) and an iron-removed quantity of 11.9 g. The sister (female 3) presented a mild biological iron overload, only marked by increasing transferrin saturation (73%). However, it should be noted that she was identified using family screening.

Thus, in the absence of large population studies and without more relevant hemojuvelin functional analysis, it is not possible to identify which of the four new mutations detected in our cohort of 310 C282Y homozygous patients are loss-of-function mutations and which are potential uninformative polymorphisms. However, with the exception of female 3, the comparative studies presented here of C282Y homozygous males and females of similar age ranges have clearly shown that iron indices of patients with an additional \( HJV \) mutation

### Table 1. Genotypes and clinical manifestations of the nine C282Y homozygous patients with an additional \( HJV \) mutation

<table>
<thead>
<tr>
<th>Patients</th>
<th>( HJV ) gene mutation</th>
<th>Nucleic acid change</th>
<th>Amino acid change</th>
<th>Frequency on the 666 control chromosomes</th>
<th>Clinical manifestations</th>
<th>Arthropathy</th>
<th>Skin pigmentation</th>
<th>Heart disease</th>
<th>Hepatic fibrosis</th>
<th>HIS</th>
<th>Phlebotomy IR (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male 1</td>
<td>302 CTC&gt;CCC</td>
<td>L101P</td>
<td>2/666</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>NP</td>
<td>NA</td>
<td>22.8</td>
<td>&gt;4.6</td>
<td></td>
</tr>
<tr>
<td>Male 2</td>
<td>302 CTC&gt;CCC</td>
<td>L101P</td>
<td>2/666</td>
<td>+</td>
<td>NA</td>
<td>2.4</td>
<td>5.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male 3</td>
<td>904 GAG&gt;AAG</td>
<td>E302K</td>
<td>1/666</td>
<td>-</td>
<td>-</td>
<td>NA</td>
<td>2.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male 4</td>
<td>1114 AAC&gt;GAC</td>
<td>N372D</td>
<td>0/666</td>
<td>-</td>
<td>-</td>
<td>NP</td>
<td>2.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male 5</td>
<td>1004 CGG&gt;CAG</td>
<td>R335Q</td>
<td>0/666</td>
<td>-</td>
<td>+</td>
<td>NP</td>
<td>2.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Male 6</td>
<td>904 GAG&gt;AAG</td>
<td>E302K</td>
<td>1/666</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>1.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female 1</td>
<td>314 TCG&gt;TGG</td>
<td>S105L</td>
<td>0/666</td>
<td>-</td>
<td>+</td>
<td>NP</td>
<td>2.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female 2</td>
<td>959 GGG&gt;GAG</td>
<td>G320V</td>
<td>0/666</td>
<td>+</td>
<td>+</td>
<td>NP</td>
<td>2.4</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Female 3</td>
<td>1004 CGG&gt;CAG</td>
<td>R335Q</td>
<td>0/666</td>
<td>+</td>
<td>-</td>
<td>1.5</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

HIS, (Histological) hepatic iron score; phlebotomy IR, phlebotomy iron removed; NP, (liver biopsy) not performed; NA, (liver biopsy results) not available.

*Curative phlebotomies have only just started.

### Table 2. Comparative study of iron parameters in C282Y homozygous males

<table>
<thead>
<tr>
<th>The six males with a ( HJV ) mutation</th>
<th>Cohort of the C282Y homozygous males*</th>
<th>Age-D (years)</th>
<th>SF (( \mu \text{g/l} ))</th>
<th>TS (%)</th>
<th>Age range (years)</th>
<th>No. of patients</th>
<th>Means of SF (( \mu \text{g/l} ))</th>
<th>% of SF values greater than those of males with a ( HJV ) mutation</th>
<th>Means of TS (%)</th>
<th>% of TS values greater than those of males with a ( HJV ) mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male 1</td>
<td></td>
<td>23</td>
<td>2000</td>
<td>85</td>
<td>15–25</td>
<td>3</td>
<td>396 ± 159</td>
<td>0.0 (0/3)</td>
<td>87.0 ± 7.6</td>
<td>66.7 (2/3)</td>
</tr>
<tr>
<td>Male 2</td>
<td></td>
<td>30</td>
<td>4800</td>
<td>85</td>
<td>25–35</td>
<td>23</td>
<td>946 ± 802</td>
<td>0.0 (0/23)</td>
<td>77.6 ± 12.6</td>
<td>30.4 (7/23)</td>
</tr>
<tr>
<td>Male 3</td>
<td></td>
<td>32</td>
<td>598</td>
<td>94</td>
<td>60.9 (14/23)</td>
<td>16</td>
<td>886 ± 793</td>
<td>5.7 (3/53)</td>
<td>79.9 ± 14.1</td>
<td>43.4 (23/53)</td>
</tr>
<tr>
<td>Male 4</td>
<td></td>
<td>38</td>
<td>1960</td>
<td>85</td>
<td>35–45</td>
<td>53</td>
<td>886 ± 793</td>
<td>5.7 (3/53)</td>
<td>79.9 ± 14.1</td>
<td>43.4 (23/53)</td>
</tr>
<tr>
<td>Male 5</td>
<td></td>
<td>42</td>
<td>2657</td>
<td>85</td>
<td>3.8 (2/53)</td>
<td>16</td>
<td>1018 ± 493</td>
<td>0.0 (0/16)</td>
<td>85.3 ± 13.0</td>
<td>31.3 (5/16)</td>
</tr>
<tr>
<td>Male 6</td>
<td></td>
<td>68</td>
<td>2624</td>
<td>95</td>
<td>65–75</td>
<td>16</td>
<td>1018 ± 493</td>
<td>0.0 (0/16)</td>
<td>85.3 ± 13.0</td>
<td>31.3 (5/16)</td>
</tr>
</tbody>
</table>

Age-D, Age at diagnosis; SF, serum ferritin; TS, transferrin saturation.

*Excluding those with a \( HJV \) or \( HAMP \) gene mutation.

### Table 3. Comparative study of iron parameters in C282Y homozygous females

<table>
<thead>
<tr>
<th>The three females with a ( HJV ) mutation</th>
<th>Cohort of the C282Y homozygous females*</th>
<th>Age-D (years)</th>
<th>SF (( \mu \text{g/l} ))</th>
<th>TS (%)</th>
<th>Age range (years)</th>
<th>No. of patients</th>
<th>Means of SF (( \mu \text{g/l} ))</th>
<th>% of SF values greater than those of females with a ( HJV ) mutation</th>
<th>Means of TS (%)</th>
<th>% of TS values greater than those of females with a ( HJV ) mutation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Female 1</td>
<td></td>
<td>49</td>
<td>586</td>
<td>96</td>
<td>45–55</td>
<td>32</td>
<td>542 ± 641</td>
<td>18.8 (6/32)</td>
<td>76.1 ± 15.0</td>
<td>9.4 (3/32)</td>
</tr>
<tr>
<td>Female 2</td>
<td></td>
<td>50</td>
<td>1232</td>
<td>93</td>
<td>9.4 (3/32)</td>
<td>32</td>
<td>542 ± 641</td>
<td>18.8 (6/32)</td>
<td>76.1 ± 15.0</td>
<td>9.4 (3/32)</td>
</tr>
<tr>
<td>Female 3</td>
<td></td>
<td>53</td>
<td>97</td>
<td>73</td>
<td>93.8 (30/32)</td>
<td>32</td>
<td>542 ± 641</td>
<td>18.8 (6/32)</td>
<td>76.1 ± 15.0</td>
<td>9.4 (3/32)</td>
</tr>
</tbody>
</table>

Age-D, Age at diagnosis; SF, serum ferritin; TS, transferrin saturation.

*Excluding those with a \( HJV \) or \( HAMP \) gene mutation.
were amongst the most elevated. In males, the results were also significant \((P = 0.0233,\) Student’s \(t\)-test) for the serum ferritin concentrations. These data led us to propose that both juvenile haemochromatosis genes could explain one part of the C282Y/C282Y-related phenotypic heterogeneity by accentuating the iron burden. As recently reported for the HAMP gene \((10),\) the use of HFE knockout mice \((Hfe^{-/-})\) with an additional deficient allele of the HJV gene \((Hjv^{+-/-})\) would help us to confirm the HJV modifier gene effect.

A digenic mode of inheritance, on the basis of heterozygous mutations in both the HFE and HAMP genes, has recently been proposed to explain iron overload in patients from different origins \((19–21).\) Another objective of the present study was to determine if HJV mutations could contribute to the expression of an iron overload phenotype in patients with at least one chromosome lacking a HFE mutation. To address this second question, we scanned the HJV coding region in a cohort of 31 previously described patients \((11),\) and no mutations were found (data not shown). We are currently continuing our investigation with other patients. A positive result would strengthen the link between existence of mutations at both the HFE and HJV loci and iron overload severities. Another possibility would be to explore family members of the two R335Q carriers (male 5 and female 3).

To conclude, our study illustrates the complexity of the genotype/phenotype correlations in what was previously thought to be a monogenic disorder, as it is clear that gene–gene interactions modify phenotypic expression of the disease.

**MATERIALS AND METHODS**

**Patients and controls**

Informed consents were obtained from the C282Y homozygous patients and 333 control subjects.

The C282Y homozygous patients were enrolled in the study because they had a transferrin saturation level \(\geq 45\%\). At the first visit of these patients to a blood center in Brittany (western part of France), a clinical questionnaire was completed by the specialized physician. Information contained in this questionnaire was previously described in detail \((15).\) It notably provided information regarding socio-demographic characteristics of patients, their age at onset, the circumstances of HH discovery, the biochemical parameters and the clinical signs associated at the time of onset. This questionnaire also included data related to the treatment, such as the number and quantity of phlebotomies needed to reach depletion and the quantity of iron extracted.

The control subjects consisted of bone marrow donors from the same geographical area, and presented normal iron indices.

**HJV mutation analysis**

**PCR primers and DNA amplification for DHPLC and sequencing analysis.** For DHPLC scanning of the whole HJV coding region (including intron–exon junctions) we designed three sets of primers on the basis of the GenBank NT_004434 sequence. Coding exon 2 was amplified using the forward primer 5’-AAGAACCGCCGCTTG-3’ and the reverse primer 5’-TCCCCAAACCCTTCCCTG-3’, exon 3 was amplified using the forward primer 5’-GAAAACTACACTCCGCATAGAGCAT-3’ and the reverse primer 5’-GTGGCCGTGGGAAATCTCAT-3’ and exon 4 was amplified using the forward primer 5’-TTAGGCCCATGAGTCCCTG-3’ and the reverse primer 5’-TGCCCAATCTGTATCTCATA-3’. These primers were also used for the sequencing analysis.

Amplified fragments were 162–801 bp in length (Table 4). Polymerase chain reaction (PCR) were performed in a 25 \(\mu\)l reaction volume containing the following: 10 pmol of each primer, 1.0–2.0 \(\mu\)M MgCl₂, 1 x PCR buffer II (Applied Biosystem), 200 \(\mu\)M each dNTPs (Promega), 1 U of AmpliTaq DNA polymerase (Applied Biosystems) and 100 ng of DNA.

A touchdown PCR protocol was used to minimize PCR optimization and to reduce the number of thermocycler programs; one protocol was enough to amplify all three coding exons. Cycling conditions were as follows: a denaturation step at 94°C for 3 min; seven touchdown cycles with annealing temperature decreasing of 1°C per cycle from 63 to 57°C (denaturation at 94°C for 30 s, annealing for 30 s, primer extension at 72°C for 45 s); 35 cycles with a 56°C annealing temperature; and a final elongation step at 72°C for 5 min. All reactions were carried out using the GeneAmp PCR system 9700 (Applied Biosystem).

**DHPLC analysis and sequencing.** DHPLC analysis was carried out using the WAVE® DNA Fragment Analysis System (Transgenomic). The DHPLC melting profile and analytical conditions for each DNA fragment were first predicted using WaveMaker® software (Transgenomic) and then analytical conditions were established on the basis of experimentally determined melting curves. Heteroduplexes were formed by heat denaturation of PCR products at 94°C for 3 min and then cooling to 68°C for 15 min. DHPLC conditions are shown in Table 4.

Sequencing of the DHPLC positive samples was performed using a fluorescent-tagged dideoxy chain termination method with a MegaBACE 1000 DNA sequencing system (Amersham).

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