HAMP AS A MODIFIER GENE THAT INCREASE THE PHENOTYPIC EXPRESSION OF THE HFE p.C282Y HOMOZYGOUS GENOTYPE

Running title: modifier genes in hereditary hemochromatosis type I

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* The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint first authors.

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ABSTRACT

Hereditary hemochromatosis is a genetically heterogeneous disease of iron metabolism. The most common form of the disorder is an adult-onset form which has mainly been associated with the \textit{HFE} \texttt{p.C282Y/p.C282Y} genotype. The phenotypic expression of this genotype is very heterogeneous and could be modulated by both environmental factors and modifier genes. The non-\textit{HFE} hereditary hemochromatosis forms include a juvenile onset form associated with mutations in \textit{HAMP}. From a cohort of 392 \texttt{C282Y} homozygous patients, we found 5 carriers of an additional \textit{HAMP} mutation at the heterozygous state (\texttt{p.R59G, p.G71D} or \texttt{p.R56X}). We found that iron indices of these five patients were among the most elevated of the cohort. Moreover, we specified that the \textit{HAMP} mutations were not detected in 300 control subjects. These results revealed that mutations in \textit{HAMP} might increase the phenotypic expression of the \texttt{p.C282Y/p.C282Y} genotype. From a cohort of 31 patients with at least one chromosome lacking an \textit{HFE} mutation, we further identified four males carrying a heterozygous \textit{HAMP} mutation (\texttt{p.59G or p.G71D}). Based on a digenic model of inheritance, these data suggest that the association of heterozygous mutations in the \textit{HFE} and \textit{HAMP} genes could lead, at least in some cases, to an adult-onset form of primary iron overload.

Abstract word count: 203.
INTRODUCTION

Hereditary Hemochromatosis (HH) is an inherited disorder of iron metabolism characterised by progressive accumulation of iron in tissues. Such an iron overload can lead to cirrhosis, diabetes mellitus, arthropathy, endocrine abnormalities, hepatocellular carcinoma and cardiomyopathy. The most common form of the disorder, called hereditary hemochromatosis of type I (OMIM # 235200), is an adult-onset form associated with duodenal iron hyperabsorption. It is inherited in an autosomal recessive pattern and has, since 1996, been mainly linked with the p.C282Y HFE gene (GenBank # Z92910) mutation. In addition to the p.C282Y mutation, which has been reported to be homozygous in 64 to 100% of HH patients of European origin, 18 other mutations have now been reported. The p.H63D and p.S65C substitutions have been particularly associated with milder iron overload phenotypes.

Non-HFE-related forms of primary iron overload have been documented in recent years. These include an autosomal recessive form caused by mutations in the Tfr2 gene (HH type III; OMIM # 604250), an autosomal dominant form due to mutations in the SLC11A3 gene (HH type IV; OMIM # 606069), another autosomal dominant form due to a mutation in the iron-responsive element of H ferritin mRNA (HH type V; OMIM # 134770) and an autosomal recessive juvenile-onset form associated with mutations in either the very recently identified 1q-related gene (HH type IIA; OMIM # 602390) or in the HAMP gene (HH type IIB; OMIM # 606464).

The HAMP gene (GenBank # AJ277280) has very recently been implicated in a juvenile form of hereditary hemochromatosis based on the description of two mutations, found in the
homozygous state, in two families: the deletion of guanine 93 (nucleotide 1 is the adenine in the initiator methionine) which results in a frameshift and the g.166 C>T transition which changes amino acid 56 from arginine to a stop codon (p.R56X) \(^{22}\).

Since the identification of the \(HFE\) gene, several studies have reported that the phenotypic expression of the p.C282Y/p.C282Y genotype is very heterogenous \(^{23, 24}\). The influence of environmental factors, such as an excessive alcohol consumption \(^{25}\), surely explains one aspect of this phenotypic heterogeneity. As recognized by different authors \(^{26-28}\), the influence of other genes, that act via the same molecular pathways as HFE, is an attractive additional explanation.

The main objective of the present study was to determine if mutations in the \(HAMP\) gene were associated with more severe iron overload phenotypes in p.C282Y homozygous patients. In five individuals we identified a \(HAMP\) gene mutation in the heterozygous state: either the p.R59G and p.G71D missense mutations, which are novel, or the previously described p.R56X nonsense mutation. We argue that iron parameters of the five p.C282Y homozygous patients with an additional \(HAMP\) mutation were effectively among the most elevated.

Based on these findings, we also searched for \(HAMP\) mutations in 31 HH patients with at least one chromosome without a \(HFE\) mutation. In one p.C282Y, two p.S65C and one p.H63D carriers, the p.R59G or the p.G71D \(HAMP\) mutation could be detected. A family study, from the p.C282Y/p.R59G proband, further confirmed that mutations in both the \(HAMP\) and \(HFE\) genes can lead to an iron overload phenotype, whereas normal iron parameters were found in subjects heterozygous for a mutation in either gene. These additional data led us to propose that, at least in some cases, the addition of a heterozygous
mutation at the both the *HFE* and *HAMP* loci could be responsible of an adult-onset iron overload phenotype.
MATERIAL AND METHODS

PATIENTS AND CONTROLS

Informed consent was obtained from all subjects including controls for DNA studies.

We first studied the HAMP coding region in 392 p.C282Y homozygous patients that had a transferrin saturation level greater than or equal to 45 percent. At the first visit of these patients to a blood center of the western part of France, a clinical questionnaire was completed by the specialised physician. Information contained in this questionnaire was previously described in detail. 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.

We also selected 31 HH patients, from a cohort of 450 treated by phlebotomy, based on three criteria: (1) a transferrin saturation level above the threshold (i.e. ≥ 60% in males and ≥ 50% in females), (2) at least one chromosome without a HFE mutation (this point was checked by a complete D-HPLC scanning of the HFE gene, as previously described), and (3) exclusion of secondary known causes of iron overload. There was no history of excess alcohol intake (< 60g daily), hematologic disease, blood transfusions or excess oral iron intake. Moreover, serological testing for hepatitis C and B were negative.
Three hundred bone marrow donors with normal iron indices, from the same geographical area, were included in the study as controls.

**HAMP MUTATIONS ANALYSIS**

*Sequence information and PCR*

For the DHPLC scanning of the whole *HAMP* coding sequence we designed two sets of primers based on the GenBank AJ277280 *HAMP* gene sequence: exon 1 was amplified using the forward primer 5’-GCCCTAAAACGACTGTC-3’ and the reverse primer 5’-CATCCCTGCTGCTGCCCTGCTA-3’, while exons 2 and 3 were co-amplified using the forward primer 5’-TCTCAGAGGTCCACTGGGC-3’ and the reverse primer 5’-GACACTCGGCAGAGAGAAAG-3’. These primers were also used for the sequencing analysis.

PCR reactions were carried out using the GeneAmp® PCR system 9700 (Applied Biosystems, Foster city CA). PCR cycles were as follows: 94°C for 5 min and 40 cycles, 94°C, 30s; 57°C or 59°C (exon 1 and exons 2-3 respectively) and 72°C, 30s.

To promote heteroduplex formation the PCR product was heated to 95°C for 5 min and then cooled to 68°C over 15 min.

**DHPLC analysis and sequencing**

DHPLC analysis was carried out using the WAVE™ DNA Fragment Analysis System (Transgenomic, San Jose city CA). The melting profile and analytical conditions for each
DNA fragment (exon 1 and exons 2-3 of the *HAMP* gene) were first predicted using WaveMaker software (Transgenomic, San Jose city CA) and then analytical conditions were established based on experimentally determined melting curves. DHPLC conditions are shown in Table 1. Sequencing of the DHPLC positive samples was performed using a fluorescent-tagged dideoxy chain termination method with an ABI Prism™ Genetic Analyser (Applied Biosystems, Foster city CA).
RESULTS

**HAMP ANALYSIS IN 392 p.C282Y HOMOZYGOUS HH PATIENTS AND 300 CONTROL SUBJECTS**

DHPLC analysis and subsequent DNA sequence analysis provide a sensitive means of identifying novel mutations in scanned genes. We used this method to analyze the *HAMP* coding region from 392 p.C282Y homozygous HH patients with mild to severe iron overloads. DHPLC analysis revealed that two sibs (a male and a female) and three non-related females were heterozygotes for a variation in the region encompassing exons 2 to 3 of the *HAMP* gene. Subsequent sequencing analysis identified a cytosine to guanine transition at position 175 (nucleotide 1 is the adenine in the initiator methionine) in two non-related females which changes amino acid 59 from arginine to glycine (p.R59G) (Figure 1A), a guanine to adenine transition at position 212 in another female which changes amino acid 71 from glycine to aspartic acid (p.G71D) (Figure 1B), and the previously described p.R56X *HAMP* mutation in the two sibs.

None of the three p.R56X, p.R59G and p.G71D *HAMP* mutations were detected in a control group of 300 individuals from the same geographical area.


*HAMP* encodes for a pro-peptide of 84 amino acids (SwissProt # P81172), called the hepcidin precursor protein, that undergoes enzymatic cleavage into mature peptides of 20, 22 and 25 amino acids. As illustrated in Figure 2, arginine 59 is at the critical position of a penta-arginine basic domain (residues 55-59) from which the cleavage processing occurs (the furin
pro-hormone convertase is probably implicated in this biological activity\textsuperscript{30).} Glycine 71 is located between four of the eight cysteines that, based on the structure recently defined by HN. Hunter and co-workers using H MNR spectroscopy\textsuperscript{31}, form intra-molecular bonds and stabilize the \(\beta\)-sheet structure of the 25-amino acid mature peptide.

**IRON OVERLOAD PHENOTYPES OF THE 5 p.C282Y HOMOZYGOUS HH PROBANDS THAT HAVE INHERITED AN ADDITIONAL MUTATION IN THE \textit{HAMP} GENE**

The male who carried the \textit{HAMP} p.R56X mutation presented with a severe iron overload phenotype as indicated by a transferrin saturation of 89%, a serum ferritin of 2242 µg/L and a quantity of iron removed by phlebotomy of 14.6g. Despite this significant iron reduction, normal iron indices have not been reached at the time of this report. His transferrin saturation level is still elevated (56%), while serum ferritin is normal (38 µg/L).

As shown in Table 2, iron overload phenotypes observed in the four females appeared to be more severe than those found in p.C282Y homozygous females of similar age ranges. In particular we found that the mean transferrin saturation level of the four females was significantly different from that of p.C282Y homozygous females (age range at diagnosis: 45 to 75 years) without a \textit{HAMP} gene mutation (92.5 [\(\sigma = 259.3\)] vs. 74.3 [\(\sigma = 259.3\)] percent; \(p = 0.0326\), Student-\(t\) test). A similar statistical analysis was performed on the serum ferritin means, using a logarithmic transformation in order to normalize the values, but no significant difference was obtained (644.0 [\(\sigma = 259.3\)] vs. 661.8 [\(\sigma = 1098.3\)] µg/L; \(p = 0.3530\), Student-\(t\) test).
These results led us to take into account the fact that HAMP gene mutations could also contribute to the expression of an iron overload phenotype in HH patients with at least one chromosome without a HFE mutation.

HAMP DHPLC IN HH PATIENTS WITH AT LEAST ONE CHROMOSOME WITHOUT A HFE MUTATION

The entire coding region of the HAMP gene was screened in 31 selected HH patients with at least one chromosome lacking a HFE mutation (details of the selection criteria are provided in the Materials and Methods section). In four male carriers of one mutation at the HFE locus (either the p.C282Y, p.H63D or p.S65C) the p.R59G or p.G71D missense mutations could be detected at the HAMP locus. Details of patient genotypes as well as of their biochemical iron parameters assayed at the time of diagnosis are provided in Table 3.

Venesections were instituted in the four HH patients. An equivalent of 7.4g of iron, sufficient to restore normal iron status, was removed in one p.S65C/p.R59G carrier (the 50-year-old man), whereas an equivalent of 9g of iron was removed in the p.H63D/p.G71D carrier. In the other two males the curative part of the venesection process has begun more recently and is still in course. Consequently, quantities of iron removed are not yet informative.

Family study

For the p.C282Y/p.R59G mutations carrier, a rapid family study was feasible. The family tree is present in Figure 3.
The proband (number II.1) was a 43-year-old man with a transferrin saturation of 70% and ferritin of 849 µg/L. Interestingly, his mother was already known to display the p.C282Y homozygous genotype. She was included in a venesections protocol at our center during which an equivalent of 8g of iron was removed in order to restore normal iron indices. The proband’s father was found to be heterozygous for the p.R59G HAMP mutation and had normal iron parameters.

The proband has two brothers and two sisters. The younger brother (II.4) had an identical genotype with a transferrin saturation of 63% and serum ferritin of 360 µg/L. As for the proband, he has no other known cause of iron disorder. The second brother (II.3) and one sister (II.5) were found to be heterozygous for the p.C282Y HFE and p.R59G HAMP mutations without evidence of an iron overload. It must be emphasized that the woman gave birth three months prior to our determination of iron parameters and that, before this event, she had a transferrin saturation of 63% (data collected from another biological laboratory). The second sister (II.2) was heterozygous for the p.C282Y HFE mutation with normal transferrin saturation and serum ferritin levels.
DISCUSSION

The phenotypic expression of the p.C282Y homozygous genotype is very heterogeneous and could be modulated by both environmental factors and modifier genes. To address the question of whether HAMP could be one such gene, we performed DHPLC scanning of the HAMP coding region, with subsequent sequence analysis, in 392 p.C282Y homozygous patients. In five of these patients, one male and four females, we identified three HAMP mutations in the heterozygous state. Two of these mutations were novel, the p.R59G and p.G71D missense mutations, and one was previously described, the p.R56X nonsense mutation.

Positional study of the p.R59G amino acid change revealed that it occurred in a pentar-arginine basic domain (residues 55-59) in which arginine 59 is at the critical position for activity of pro-hormone convertases and, particularly, for furin which processes substrates having a RX(K/R)R sequence. Functionally, one may thus consider that the effect of this mutation is to prevent the formation of the 25-amino acid mature peptide. The example of mutations that change the last arginine of the fibroblast growth factor 23 (FGF23) RXXR processing site, which are thought to prevent a proteolytic cleavage activity and result in the Autosomal Dominant Hypophosphataemic Rickets (ADHR; OMIM # 193100) disorder, argue for this assumption. To date, the functional relevance of the p.G71D amino acid substitution is less clear. However, it must be emphasized that this missense mutation is located between four of the eight structural cysteines of the 25-amino acid mature peptide and that the change of a neutral amino acid to an acidic residue frequently leads to crucial protein structure modifications.
The comparative study presented here of p.C282Y homozygous females of similar age ranges has shown that iron indices of the four females with a HAMP mutation were among the most elevated. The results were also significant (p = 0.0326, Student-\(t\) test) for the transferrin saturation levels. The male who carried the HAMP p.R56X mutation harbored a more severe iron overload phenotype (transferrin saturation of 89%, serum ferritin of 2242 µg/L and a quantity of iron removed by phlebotomy of 14.6g) and, while his serum ferritin concentration is now normal (38 µg/L), in spite of 55 phlebotomies his transferrin saturation level is still clearly above the normal range (56%). These data led us to propose that, even if they are not frequent 37-39, HAMP mutations could effectively explain one part of the p.C282Y/p.C282Y-related phenotypic heterogeneity by accentuating the iron burden.

Based on these findings, another objective of our study was to determine if HAMP mutations could also contribute to the expression of an iron overload phenotype in a group of 31 adult-onset patients with at least one chromosome lacking a HFE mutation. These patients were identified in a screening study based on an elevated transferrin saturation level (≥ 60% in males and ≥ 50% in females) in the absence of other known causes of iron disorder. In 4 of the 31 HH selected patients (i.e. 13%), we identified the two novel p.R59G and p.G71D missense mutations in the heterozygous state.

We further performed a family study in relatives of the p.C282Y and p.R59G mutations carrier. The proband had a transferrin saturation level of 70% and a serum ferritin level of 849 µg/L at diagnosis. His younger brother (II.4), who is also a carrier of the p.C282Y and p.R59G mutations, had a transferrin saturation level of 63% and a serum ferritin level (360µg/L), which is clearly above the normal range for a 34-year-old man. The proband’s
father and one of his sisters, who are respectively heterozygous for the p.R59G and p.C282Y mutations, had normal transferrin saturation and serum ferritin levels.

Taken together, these data confirm that the combination of a genetic alteration at both the \textit{HFE} and \textit{HAMP} loci could lead to an adult-onset phenotype of hereditary hemochromatosis that is not apparent when an individual carries only one of these gene alterations. A similar situation has very recently been reported by Merryweather-Clarke \textit{et al} who have partially described the pedigree of a proband heterozygous for the C282Y \textit{HFE} mutation and for a \textit{HAMP} deletion that removes the last three nucleotides of exon 2 and the first one of intron 2\textsuperscript{33}. Thus, based on the example of several other genetic disorders\textsuperscript{40}, it is tempting to propose that, at least in some cases, a digenic model of inheritance might be responsible for an adult-onset iron overload phenotype.

Observation that the older brother of the proband (II.3) had normal iron parameters led us to postulate that the penetrance of the p.C282Y/p.R59G genotype might, however, not be complete. This might be related to the recognition of non-expressing elderly p.C282Y homozygous individuals, even with relatives of iron overload p.C282Y/p.C282Y patients\textsuperscript{41,42}. On the other hand, one can also consider a more complex genetic situation implicating a third mutation which might be located either in non-coding regions of the \textit{HFE} and \textit{HAMP} genes or in another iron metabolism gene. Considering the familial tree of the p.C282Y/p.R59G proband, this third mutation could be detected in the proband (II.1), his younger brother (II.4) and his younger sister (II.5), but not in his older brother (II.3). Today, because non-genetic factors account for part of the iron overload processing, this later subject must be considered as carrying mutations which may lead to hemochromatosis but not yet having the disease. He needs to be followed carefully.
Our results shed new light on the molecular basis of primary iron overloads. They are in accordance with the demonstration by G. Nicolas et al of an increased hepatic iron loading in $Hfe^{-/-}$ mice with an additional $HAMP$ deficient allele (see jointly submitted manuscript). They are also in accordance with several studies that, during the past two years, have concluded that the $HAMP$ gene product, called hepcidin, might act through the same regulatory pathways as HFE to control the amount of iron in the body $^{28, 43-46}$. In the case of an excess of iron in the body, the control process should include a form of action on the reticuloendothelial macrophages, by promoting inhibition of iron release from senescent red blood cells $^{30, 47}$, and an action on duodenal cells, by promoting inhibition of alimentary iron absorption $^{30, 48, 49}$. Even if the relationship between HFE and HAMP has still to be clearly defined, it is interesting to note that the first demonstration of a relation between the two proteins has very recently been forthcoming from three studies which have highlighted that HFE plays an important role in the regulation of hepatic $HAMP$ gene expression $^{44-46}$. 
REFERENCES


TABLE 1

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<td>Age ranges (years)</td>
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<td>Means of $\text{TS} (%)$</td>
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TABLE 3

<table>
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<tr>
<td>M</td>
<td>34</td>
<td>+/-</td>
<td>+/-</td>
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FIGURE 1

A

B
FIGURE 2

MALSSQWACGLLLLALSLTSGVRPGQGRQPQQGARASWMMPFGRRRTPRCCGGCCHRSCGGMCCT

Cleavage site

Mature peptide
FIGURE 3

<table>
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<th>Years</th>
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<td>C282Y / Wt</td>
<td>R59G / Wt</td>
<td>102</td>
<td>39</td>
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</table>
TABLE AND FIGURE LEGENDS

**Table 1.** D-HPLC temperature and gradient conditions for *HAMP* coding fragments, with a flow rate of 0.9mL/min and buffer B increased at 2% per min.

**Table 2.** Comparative study of iron indice parameters p.C282Y homozygous females.

* : Excluding those with a *HAMP* gene mutation.

SF: Serum Ferritin

TS: Transferrin Saturation

Age-D: Age at diagnosis

Phlebotomy IR: Phlebotomy Iron Removed

**Table 3.** Genotypes and biochemical iron parameters from four patients with *HFE* and *HAMP* mutations.

SF: Serum Ferritin.

TS: Transferrin Saturation.

Age-D: Age at diagnosis.

**Figure 1.** Part A displays the overlay of chromatograms showing *HAMP* g.175G>C sequence alteration and wild type and the respective sequences. Part B shows the second alteration profile of *HAMP* g.212G>A and sequences.

**Figure 2.** Peptide sequence of the hepcidin precursor protein and the position of the changed amino acids.
Figure 3. Family tree of the p.C282Y/p.R59G proband.

Wt: wild type allele.

Grey square or circle: patients with a HH phenotype.

SF: Serum Ferritin.

TS: Transferrin Saturation.
EXPRESSION OF THE HFE p.C282Y HOMOZYGOUS GENOTYPE

Claude FEREC

HAMP AS A MODIFIER GENE THAT INCREASE THE PHENOTYPIC
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