

# Mutations in HAMP and HJV genes and their impact on expression of clinical hemochromatosis in a cohort of 100 Spanish patients homozygous for the C282Y mutation of HFE gene

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**Abstract** Most hereditary hemochromatosis (HH) patients are homozygous for the C282Y mutation of the HFE gene. Nevertheless, penetrance of the disease is very variable. In some patients, penetrance can be mediated by concomitant mutations in other iron master genes. We evaluated the clinical impact of hepcidin (HAMP) and hemojuvelin mutations in a cohort of 100 Spanish patients homozygous for the C282Y mutation of the HFE gene. HAMP and hemojuvelin mutations were evaluated in all patients by bidirectional direct cycle sequencing. Phenotype–genotype interactions were evaluated. A heterozygous mutation of the HAMP gene (G71D) was found in only one out of 100 cases. Following, we performed a study of several members of that family, and we observed several members had a digenic inheritance of the C282Y mutation of the HFE gene and the G71D mutation of the HAMP gene. This mutation in the HAMP gene did not modify the phenotype of the

individuals who were homozygous for the C282Y mutation. One other patient presented a new polymorphism in the hemojuvelin gene, without consequences in iron load or clinical course of the disease. In conclusion, HAMP and hemojuvelin mutations are rare among Spanish HH patients, and their impact in this population is not significant.

**Keywords** HFE · Hepcidin · HAMP · Hemojuvelin · Hemochromatosis · Iron metabolism

## Introduction

Hereditary hemochromatosis (HH) is characterised by impaired iron homeostasis, leading to an increase in intestinal iron absorption and excessive accumulation in tissues. If the disorder is not treated, the build-up of iron can cause tissue damage and may lead to cirrhosis, diabetes mellitus, arthropathy, cardiomyopathy, endocrine abnormalities and hepatocellular carcinoma [19]. The vast majority of cases of clinical hereditary hemochromatosis are associated with homozygosity for the C282Y HFE mutation (HH type 1) [9]. The penetrance of the disease is very variable. HH is generally considered a genetic condition with high prevalence but low morbidity [1, 5]. Concomitant genetic and environmental factors may contribute to differential expression of C282Y homozygosity.

Other forms of hemochromatosis include type 2 or juvenile hemochromatosis, which can be caused by mutations in the HAMP, the gene coding for the iron regulatory peptide hepcidin [21] or in the hemojuvelin gene [18]. Type 3 is caused by mutations in the transferrin receptor 2 gene [8], and type 4 is described as an autosomal

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dominant iron overload that develops from a pathogenic mutation in the SLC40a1 gene (ferroportin disease) [16, 17, 20]. Some authors have described mutations in HAMP and HJV as genetic modifiers of the hemochromatosis phenotype in humans [10, 12, 15]. The objective of this study was to determine the impact of alterations in HAMP and HJV genes on the phenotype of homozygous patients for the C282Y mutation, in a clinically well-defined group of 100 Spanish HH probands [3].

## Materials and methods

All patients selected for this study were referred to the hematologist for biochemical evidence of iron overload, with or without symptoms compatible with genetic hemochromatosis. Transferrin saturation and serum ferritin were measured at diagnosis by standard methods in samples obtained after an overnight fast. In those patients with a transferrin saturation index  $\geq 55\%$  or a serum ferritin level  $\geq 400$   $\mu\text{g/L}$  on two occasions, HFE mutations were analysed using the LightCycler<sup>®</sup> 2.0 Real-Time Polymerase Chain Reaction (PCR) System [2] (Roche Diagnostics GmbH, Mannheim, Germany). One hundred non-related homozygous C282Y patients were finally included in the study. All patients were diagnosed between 1985 and 2005. The total amount of iron removed was calculated as the number of phlebotomies (with 450 mL of blood drawn at each session) multiplied by 0.2 (the number of grams of iron removed per session). The hepatic iron concentration and the hepatic iron index were determined in 22 patients with evidence of liver disease [4]. All patients gave their written informed consent to participate in the study, in accordance with the protocol approved by the ethics committee at Sant Pau Hospital.

*Definition of clinical complications* To study the association between genetic polymorphisms and body iron stores, the median value of iron removed by phlebotomies and the median value of serum ferritin were used as threshold. To evaluate the clinical severity of the disease in each patient, we considered only two clinical complications classically related with hemochromatosis: liver damage and diabetes. Liver damage was diagnosed when aspartate transaminase and alanine aminotransferase enzymes were above normal limits at least twice at intervals of over 3 months or when there was histological evidence of liver fibrosis/cirrhosis. Patients considered diabetic were those receiving treatment for diabetes and those with a fasting plasma glucose test  $\geq 126$  mg/dL on two occasions. Alcohol consumption was assessed from patient history, and  $>80$  g/day of ethanol was considered excessive. Viral serology data for hepatitis B and C were available in all patients.

## Mutational analysis

Genomic DNA was extracted from peripheral blood leukocytes using the FlexiGene DNA Kit (Qiagen, West Sussex, UK) in accordance with the manufacturer's instructions. Mutation detection was performed by PCR amplification of each of the three coding exons of the hepcidin gene (GeneBank No NM-021175) and the four coding exons of hemojuvelin gene (GeneBank no NM-213653), including intron–exon junctions. The oligonucleotide primers used for PCR amplification and the PCR conditions of both genes are listed in Table 1. After purification with Qiaquick<sup>®</sup> PCR Purification Kit (Qiagen, West Sussex, UK), PCR products were subjected to bidirectional direct cycle sequencing using the BigDye<sup>®</sup> Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems, Warrington, UK) and an ABI PRISM<sup>®</sup> 3100-Avant Genetic Analyzer (Applied Biosystems). Sequencing primers were the same as those used for PCR amplification. The identified alterations were confirmed by additional sequencing of independently amplified PCR products from the same patients.

## Results

Biochemical and clinical characteristics of the whole cohort have been described in detail elsewhere [3]. In summary, there were 73 men and 27 women, and the median age was 45 years (range 16–73). Fourteen patients consumed more than 80 g/day of alcohol, and three had a positive serology for hepatitis C virus. The median ferritin level for the whole group was 861.5  $\mu\text{g/L}$  (range 17–10,000), and the median quantity of iron removed by phlebotomy was 4.25 g (range 0.5–20). The iron removed was  $\geq 5$  g in 43 patients. A liver biopsy was performed in 33 of 44 patients who presented with liver damage. Fibrosis-cirrhosis of the liver was finally diagnosed in 28 and liver cancer in four. Twenty-four patients had diabetes. Table 2 summarises the biochemical characteristics of this cohort.

## HAMP mutations

The HAMP gene was normal in all but one case, a 61-year-old patient who presented with the G71D mutation in heterozygosity (patient III, Table 3). His transferrin saturation level was high (82%), and he had the highest level of ferritin in the family (2,500  $\mu\text{g/L}$ ). He suffered liver disease, with an abnormal level of liver enzymes. A liver biopsy showed portal fibrosis without cirrhosis. About 9 g of iron was removed by phlebotomy. Three of the proband's four brothers were studied. The fourth had died some years earlier (patient II, Table 3). Two of the three living brothers

**Table 1** Oligonucleotides and conditions used for polymerase chain reaction

Gene	Exon	Primer sequence (5′–3′)	References
HAMP	1F	GCC CCA TAA AAG CGA CTG TC	This work
	1R	CAT CCC TGC CCT GTA	This work
	2F	CAG TCT CAG AGG TCC ACT	[22]
	2R	AAT GTG AGC AGG GAA CC	[22]
	3F	TGC TCA CAT TCC CTT CCT TC	[14]
	3R	CAA GAC CTA TGT TCT GGG GC	[14]
HJV	1F	CCG TCA ACT CAG TAG CCA	[11]
	1R	CAT TTG GAC GAG AGA CAT C	[11]
	2F	CCC CAA ATT CCA GTC TGT T	[11]
	2R	CTC ATT CAG GCT CAC ATG C	[11]
	3F	CTC CGA TAG AGC AGA GGT C	[11]
	3R	GGA GCA TTG CTG TTG AAT AG	[11]
	4aF	TAG TCC TGC ATC TCT ACT TGG	[13]
	4aR	TGC AGG TCC TGT TCA GCT G	[13]
	4bF	GCT CTC CTT CTC CAT CAA GG	[13]
	4bR	CTA GTA ATG GGA CTG ATG GTC CC	[13] Modified
	4cF	GTT CTG TGG CTT TGC ATT CAG	This work
	4cR	TCA TGT CTT CTG CTT TCA GCT C	This work
	4dF	TAA GTT TAG AGG TCA TGA AGG	This work
	4dR	GCC CTC TTT CAG TGG AGT G	This work

were homozygous for the C282Y mutation of the HFE gene, and the other was heterozygous for this mutation. One of the YY brothers (I) was a 42-year-old man who had a positive serology for hepatitis C virus. He had a wild type HAMP gene. His transferrin saturation (TS) and ferritin levels were high (93% and 1,400 µg/L, respectively), liver enzymes were abnormal, and a liver biopsy showed portal fibrosis without cirrhosis. The total iron removed by phlebotomies in this patient was 10 g. The second YY brother (IV) was a 68-year-old man, with a wild-type HAMP gene. He had a long-term history of severe hematuria due to a bladder neoplasia at the time of diagnosis. For this reason, data about his serum ferritin and iron removed by phlebotomies were not suitable to evaluate aggressiveness of the hemochromatosis disease. The oldest brother (patient V) was a 71-year-old man who was heterozygous for the C282Y mutation of the HFE gene. He had the same G71D mutation on the HAMP gene as the proband. Despite this digenic inheritance, his iron metabolism was normal, and he had not been submitted to any phlebotomy procedure.

Eleven offspring (five men, six women) were also evaluated. None of them were homozygous for the C282Y mutation. One individual was compound heterozygous for the C282Y (CY) and the H63D mutations, eight were heterozygous for the C282Y mutation, one was only heterozygous for the H63D mutation and only one individual had a normal HFE genotype. Five offspring were heterozygous for the G71D mutation of HAMP gene, three in combination with a C282Y heterozygous mutation, one in combination with a heterozygous H63D mutation and one without any HFE mutation. No siblings showed features of iron overload. Iron deficiency was demonstrated in three cases, two of which were double heterozygous for C282Y and G71D mutations in HFE and HAMP genes, respectively (cases IIa and IIIc, Table 3).

#### Hemojuvelin mutations

Only one of the 100 patients studied had a non-described polymorphism in the HJV gene in heterozygosity. The

**Table 2** Biochemical characteristics of the cohort of 100 homozygous C282Y patients

Parameter	Mean	Median	Range
Transferrin saturation (%)	82	84	30–161
Ferritin (µg/L)	1,259	861	17–10,000
Iron removed (g)	5.5	4.2	0.5–20
Liver iron concentration <sup>a</sup> (N=22)	221	192	21–456
Hepatic iron index <sup>b</sup> (N=22)	4.6	4.5	0.43–8.75

<sup>a</sup> µMol/g liver dry weight

<sup>b</sup> µMol/g liver dry weight/age in years

**Table 3** Clinical characteristics of a family which was homozygous for the C282Y mutation of the HFE gene and heterozygous for the G71D mutation of HAMP gene

Case	Age/ sex	HFE	HAMP	HB (g/L)	MCV fl	SAT (%)	Ferritin ( $\mu$ g/L)	Glucose	Liver enzymes	Iron removed	Other
I	42/M	YY/HH	GG	172	93	93	1,400	Normal	Abnormal	10 g	HCV + . Portal fibrosis
Ia	35/F	CY/HH	GG	135	90	30	32	Normal	Normal	–	
Ib	37/F	CY/HH	GG	129	87	32	69	Normal	Normal	–	
Ic	43/F	CY/HH	GG	140	86	21	68	Normal	Normal	–	
II	–/M	–/–	–	–	–	–	–	–	–	–	EXITUS
IIa	33/M	CY/HH	GD	137	83	18	7	Normal	NORMAL	–	
IIb	40/F	CY/HH	GG	156	87	56	92	Normal	Normal	–	
III	61/M	YY/HH	GD	156	86	82	2,500	Normal	Abnormal	9 g	Portal fibrosis
IIIa	26/M	CY/HH	GG	154	101	32	57	Normal	Normal	–	
IIIb	27/M	CY/HH	GD	150	86	25	168	NORMAL	Normal	–	
IIIc	36/M	CY/HH	GD	126	73	10	4	Normal	Normal	–	
IV	68/M	YY/HH	GG	150	76	73	359	Abnormal	Normal	3 g	Bladder neoplasia
V	71/M	CY/HH	GD	148	92	33	86	Normal	NORMAL	–	
Va	30/F	CC/HH	GD	134	85	18	8	Normal	Normal	–	
Vb	40/M	CY/HD	GG	154	86	34	126	Normal	Normal	–	
Vc	45/F	CC/HD	GD	137	82	22	16	Normal	Normal	–	

Cases I, II, III, IV and V were brothers. Cases Ia, Ib, Ic were sons of case I, etc.

patient was diagnosed with iron overload when he was 45 years old, after a routine medical exam. He was asymptomatic and had no familial history of hemochromatosis. He had a transferrin saturation index of 87% and a ferritin level of 454  $\mu$ g/L at diagnosis. A genetic test confirmed the C282Y homozygosity in the HFE gene. A Ser264Leu polymorphism was detected in exon 4 (TCG>TTG) of the hemojuvelin gene. He has since undergone venesections, and 4 g of iron had been extracted. He had not presented any complications associated with hemochromatosis, such as liver disease or diabetes. His family refused further genetic or medical exams.

## Discussion

Our findings demonstrate that HAMP and HJV mutations are very infrequent among Spanish HH patients homozygous for the C282Y mutation of the HFE gene. This paucity of mutations/polymorphisms precluded the projected statistical comparisons of groups described in the “Materials and methods” section. We have therefore limited the report to a description of the patients with mutated genes and their families.

Only one polymorphism of each gene was detected in our cohort of 100 patients, and there was no clear impact on iron burden or clinical behavior of disease in either case. In the family carrying a G71D mutation in the HAMP gene, the proband patient suffered a severe iron overload, but a

younger brother who did not have a mutation in the HAMP gene—although he was also C282Y homozygous—had an even higher level of iron removed (10 g). Moreover, three offspring were double heterozygous for C282Y and G71D mutations in the HFE and HAMP genes, and none of the three had signs of iron overload. Nevertheless, two of the three, a 33-year-old man and a 36-year-old woman, suffered iron deficiency. The G71D mutation in the HAMP gene has been identified in France [10], Italy [6, 7] and UK [15]. Although this mutation modifies Gly71 which is located in between two pairs of cysteines with important structural roles, our findings, like those of previous authors [7], suggest it has only a minor, if any, effect on iron loading.

Our conclusion about the new single nucleotide polymorphism (SNP) in the hemojuvelin gene Ser264Leu is similar. The only patient we found with this polymorphism had a mild iron overload that was concordant with his C282Y homozygous condition. This SNP does not seem to influence the iron amount or clinical pattern of this individual with hemochromatosis. Our study population varied greatly. It ranged from patients with hepatic cirrhosis or liver cancer to asymptomatic individuals [3], illustrating the spectrum of the disorder. Previous studies have shown that HAMP and hemojuvelin mutations act as genetic modifiers in hemochromatosis [10, 12, 15]. Nevertheless, the very low frequency of these alterations in our patient cohort indicates a minor impact of these mutations in the penetrance of HH type 1 in Spanish patients.

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