Absence of hepcidin gene mutations in 10 Italian patients with primary iron overload

We analyzed the hepcidin gene in 10 Italian patients with hemochromatosis not related to C282Y, H63D or other less frequent HFE mutations, nor to Y250X in TFR2. The sequencing of the whole hepcidin coding region, intron-exon junctions, 5' and partially 3'UTRs, did not reveal any alteration in the studied patients.

Hereditary hemochromatosis (HH) is a common disorder of iron metabolism usually inherited as an autosomal recessive trait, although families with an autosomal dominant pattern have been described.[1]

In 1996 Feder *et al.*[2] cloned the HFE gene, the major gene responsible for HH, and found two mutations, C282Y and H63D, in HH patients, although the latter mutation seems to have a controversial role as a cause of HH. Since then it has become evident that other genes are involved in the disease. In fact, a substantial percentage of HH individuals, especially in southern Europe, do not carry the C282Y mutation. Although other alterations in the HFE gene have been described, they are present in only a minority of cases, being in large part private mutations.[3]

Three additional HH-related loci have been described: the juvenile hemochromatosis (HFE2) gene,[4] mapping in 1q, not yet identified; the transferrin receptor 2 (TFR2) gene[5] and the SLC11A3 gene,[1] encoding ferroportin, a protein involved in the outflow of iron from cells.

TFR2 mutations account for only a small number of HH cases.[6,7] SLC11A3 mutations are related to an autosomal dominant form of HH.[1] Their prevalence in sporadic HH is still to be tested.

Recently, unexpected extensive iron overload has been described in upstream stimulatory factor 2 (Usf2) gene knockout mice.[8,9] USF2 is a transcription factor involved in glucose-dependent gene regulation. Disruption of Usf2 in mice was associated with a complete lack of expression of the hepcidin gene. The hepcidin gene encodes for a peptide, exhibiting antimicrobial activity, overexpressed in iron overloaded or β_2-microglobulin knock-out mice.[8,9] In mice, as well as in humans, USF2 and hepcidin are closely linked. The absence of hepcidin expression was hypothesized to be caused by an alteration of the hepcidin gene consequent to Usf2 gene disruption,[8] and to be responsible for the progressive iron overload. It has been postulated that hepcidin could represent an iron sensor molecule acting by signaling the deposited iron content of the body to the intestinal crypt cells, thus regulating iron absorption.[9] The investigators proposed hepcidin as a novel candidate gene involved in the development of HH.

To test this hypothesis we studied the hepcidin gene in 10 hemochromatosis patients, eight of whom were negative for C282Y, H63D and other less frequent HH related mutations, while the other two patients were H63D heterozygotes.

The main features of these 10 unrelated Italian subjects are summarized in Table 1. Causes of iron overload, such as hematologic diseases, aceruloplasminemia, congenital atransferrinemia, dysmetabolic iron overload syndrome, chronic viral hepatitis B or C, history of chronic iron supplementation, heavy alcohol intake or blood transfusions, were considered exclusion criteria.

Screening of HFE and TFR2 genes was performed as previously described,[10] in order to identify the HFE C282Y, H63D, S65C, V53M, V59M, Q127H, E168X, E168Q, V169X, Q283P and

Table 1. Main data of the patients studied.

Subject N°	Age (yrs.)	Sex	Serum iron (μg/dL)	SF ng/mL	TS (%)	Increased liver enzymes	Liver histology	Perls' stain#	Hepatic MRI§	IR•	Clinical findings	Family data
1	34	M	178	1150	79	–	ND	ND	ND	+	None	Mother and brother: affected
2§	60	M	204	1057	67	+	ND	ND	ND	+	Hepatomegaly, cardiopathy	Brother: high ferritin levels
3#	69	F	281	1782	97	–	ND	ND	ND	+	None	None
4	35	M	144	904	63	+	Fibrosis	+	+	+	Hepatomegaly, hypogonadism,	None
5	37	M	197	584	72	+	Fibrosis	+	ND	–	Hepatomegaly	None
6*	44	M	229	3058	93	+	Fibrosis	+	ND	–	None	Mother: unspecified liver disease
7	52	F	216	1100	80	+	ND	ND	ND	+	Hepatomegaly, diabetes, hypogonadism	None
8	45	M	204	2892	97	+	ND	ND	+	–	Arthritis, diabetes, cardiac dysrhythmias, hypogonadism,	None
9	40	F	223	2800	88	+	Lymphocytic infiltrate	+	ND	–	None	None
10	65	M	144	643	61	–	Normal	+	ND	+	None	None

2§ is extremely obese; 3# developed pancreatic cancer in 1995; 6 is a β-thalassemia carrier (Hb = 10-11 g/dL). Perls' stain # = presence of hepatic parenchymal iron deposits; hepatic MRI§ = estimation at hepatic MRI of high iron stores; IR• = repeated phlebotomy, iron removed: >5 g (men) >3 g (women); TS = transferrin saturation; SF = serum ferritin; ND = not determined.*

Table 2. Hepcidin amplification and sequencing primers.

Exon	Sequence	Annealing temperature (°C)	Product (bp)
1F	5'-actgtcactcggtcccagac-3'	68	270
1R	5'-gagacgtcctgagctctgct-3'		
2F	5'-gagccagtctcagaggtcca-3'	68	153
2R	5'-actgtcactcggtcccagac-3'		
3F	5'-tgctcacattccccttccttc-3'	68	200
3R	5'-caagacctatgttctggggc-3'		

the TFR2 gene Y250X mutations. Eight patients did not carry any of the tested mutations, while a heterozygous H63D substitution was detected in two patients.

The whole coding region, 5' UTR, almost complete 3' UTR and exon-intron boundaries of the hepcidin gene were analyzed. Amplification was performed in a standard reaction mix. A 5%. DMSO solution was added for amplification of exons 2 and 3. Polymerase chain reaction (PCR) conditions and primers used for amplification and sequencing are described in Table 2. Direct sequencing of PCR fragments was performed on an automated sequencer (A.B.377).

DNA sequencing of the hepcidin gene revealed a wild type genotype in all examined subjects. Although we can not completely exclude the presence of rare HFE, TFR2, SLC11A3 mutations, these results indicate that, in our cases, hemochromatosis is not apparently related to hepcidin gene mutations even if a genomic alteration could be located in a regulatory region of the hepcidin gene. This hypothesis may be supported by the evidence from a mouse model in which silencing of the hepcidin gene is consequent to disruption of neighboring sequences,[8] where hepcidin regulatory regions could be present.

Alternatively, the hemochromatosis in our patients could have been caused by mutations in a different gene acting, upstream or downstream, in the same hepcidin protein pathway.

To confirm the exclusion of hepcidin as a candidate gene for rare forms of hemochromatosis further studies on additional patients are needed.

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