SCREENING HEPcidIN FOR MUTATIONS IN JUVENILE HEMOCHROMATOSIS:

IDENTIFICATION OF

A NEW MUTATION (C70R)

Short Title: A NEW HEPcidIN MUTATION

Scientific Session Heading: RED CELLS

A. Roetto¹, F. Daraio¹, P. Porporato¹, R. Caruso², T.M. Cox³, M. Cazzola⁴, P. Gasparini⁵, A. Piperno⁶, C. Camaschella¹

¹Dipartimento di Scienze Cliniche e Biologiche, Università di Torino, Azienda Ospedaliera S. Luigi, Orbassano, Torino, Italy.
²Divisione di Ematologia, IRCCS Ospedale Pediatrico Bambin Gesù, Roma, Italy.
³Department of Medicine, University of Cambridge, U.K.
⁴Dipartimento di Medicina Interna e Oncologia, IRCCS Policlinico San Matteo, Pavia, Italy.
⁵Genetica Medica, Dipartimento di Patologia Generale, II Università di Napoli and Tigem (Telethon Institute of Genetics and Medicine), Napoli, Italy
⁶Clinica Medica, Azienda Ospedaliera San Gerardo, Monza, Italy
Keywords: iron, hemochromatosis, hepcidin

Research grant support

This paper was partially supported by Telethon ONLUS Foundation Grant #GP00255Y01, European Community, Contract QLRT-1999-02237 and FIRB from Italian Ministry of Instruction Research and University to C.C.

CORRESPONDING AUTHOR

Clara Camaschella, MD
Dipartimento di Scienze Cliniche e Biologiche
Università di Torino
Azienda Ospedaliera San Luigi
10043 - Orbassano - Torino - Italy
Phone +39-011-9026837/6708175
FAX +39-011-9038636
E Mail: clara.camaschella@unito.it

Word counts

Abstract: 150
Juvenile or type 2 hemochromatosis (JH) is a genetic disease due to increased intestinal iron absorption that leads to early massive iron overload. The main form of the disease is caused by mutations in a still unknown gene on chromosome 1q. Recently, we recognized a second type of JH with clinical features identical to the 1q-linked form, caused by mutations in the gene encoding hepcidin (HEPC).

Hepcidin is an hepatic antimicrobial-like peptide whose role in iron homeostasis was first defined in animal models: deficiency of hepcidin in mice leads to iron overload, while its hepatic overexpression in transgenic animals causes iron deficiency. To define the prevalence of HEPC mutations in JH we screened for mutation HEPC gene in 21 unrelated JH subjects. We identified a new mutation (C70R), which affects one of the eight conserved cysteines that form the disulfide bonds and are critical for the stability of the polypeptide.
INTRODUCTION

Type 2 or Juvenile Hemochromatosis (JH) is a rare autosomal recessive disorder characterized by early onset and severe iron overload. The principal clinical manifestations—cardiomyopathy and hypogonadism, appear before the age of 30 years. Patients of both sexes have greatly increased transferrin saturation and serum ferritin concentrations at diagnosis and require intensive phlebotomy to achieve iron depletion.

JH is a heterogeneous genetic disorder, related to at least two distinct loci. The first maps to chromosome 1q21, but the causal gene is still unknown. A rare subset is due to mutations in hepcidin (HAMP or HEPC) gene, which maps to chromosome 19q13. As in humans, inactivation of HEPC in mouse leads to severe iron overload, whereas its overexpression in transgenic mouse leads to iron deficiency anemia. Recently, it has been demonstrated that an inappropriately low expression of HEPC mRNA is constant in HFE-related hemochromatosis, both in humans and in animal models. This implies that HFE is involved in HEPC regulation and further strengthens the concept that inability to maintain appropriate hepcidin levels is central to the development of iron excess.

Hepcidin protein shows high similarity with several cysteine-rich antimicrobial peptides. It is prevalently expressed by the hepatocytes as a precursor protein of 84 amino acids. Three active peptides originate from the propeptide by protease cleavage, respectively 25, 22 and 20 amino acids long. These soluble form of hepcidin have been isolated from the urine of normal subjects. The 25 and 20 amino acid peptides represent the major forms, while the 22 aminoacid peptide is present only at low concentration. A striking feature of the active peptides is the numerous cysteines (eight), accounting for 32% of the total amino acid content. Analysis of soluble hepcidin species for both the (20 and the 25 residues) shows that the 8 cysteines form four disulfide bonds, providing a rigid and...
tight structure to the final peptide \textsuperscript{14} (Figure 1d). Extensive promoter analysis has revealed the presence of consensus sequences for the transcription factor CEBP/\(\alpha\), that confers liver tissue specificity\textsuperscript{15}. Hepcidin synthesis is increased by iron loading and inflammation and is inhibited by iron deficiency, anemia and hypoxia\textsuperscript{16,17}.

Here we report the results of a study aimed at identifying new HEPC mutations. During the investigation of a large series of JH patients, we detected a homozygous nucleotide change that causes a missense (C70R) affecting one of the highly conserved cysteines. The mutation was found in a single patient of a consanguineous Italian family, previously considered affected by the 1q-linked type of JH.
MATERIALS AND METHOD

Twenty-one unrelated JH patients were studied. The clinical features of most cases have been previously reported\textsuperscript{18,19}. Six new cases were diagnosed using accepted criteria\textsuperscript{18}.

DNA was prepared from peripheral blood, according to standard protocols. HEPC coding sequences (NT_011109) were amplified by PCR. Primers utilised for the amplification reaction are reported in Table 1.

PCR was performed in a Thermal Cycler, using 25 pMol of each primer and 50 ng of template DNA, with an average protocol of 32 cycles (denaturation: 94°C 30”; annealing: 56°C 30”, extension: 72°C 45”) and 1 U of AmpliTaq DNA polymerase (Roche Applied Science, Indianapolis, IN, USA).

For direct sequencing, PCR products were run on 1% agarose gel, purified using QIAquick PCR purification kit (QIAGEN, Valencia, CA) and sequenced using Thermo Sequenase Cy5.5 dye terminator cycle sequencing kit. After purification from unincorporated dye with Autoseq G-50 columns, sequencing products were electrophoresed in an automatic sequencer (Applied Biosystem 373A, Foster City, CA, USA) according to manufacturer’s protocols.

Restriction endonuclease digestion was carried out using 20 µl of exon 3 PCR product and 10 U of Sac II enzyme (New England Biolabs, Inc., Beverly, MA, USA) in a final volume of 30 µl for 2 hours.
RESULTS and DISCUSSION

Most published JH families have a genetic disorder that maps to chromosome 1q\textsuperscript{2,20,21}. Homozygous mutations in HEPC gene were identified in a rare subset of JH patients, with a phenotype indistinguishable from the 1q-linked form\textsuperscript{3}. To establish the frequency of HEPC mutations in JH, we sequenced the HEPC gene of patients from 21 families, irrespective of their linkage with chromosome 1q, since linkage may be coincidental when the number of family members is small or the informativeness of the genetic markers limited. We identified a T/C mutation at the homozygous state in exon 3 (at position 208 from the starting ATG) (Figure 1a) in a young italian patient from a consanguineous family. The proband was a 11-year-old boy with high levels of transferrin saturation, serum ferritin and liver iron content, but without clinical complications. Based on the correct segregation of microsatellite alleles of chromosome 1q within the family, the disorder was previously considered 1q-linked\textsuperscript{19}. The identified nucleotide change causes the substitution of the cysteine at position 70 with arginine (C70R). Since the mutation creates a restriction site for Sac II enzyme, Sac II digestion of HEPC exon 3 was employed to demonstrate the correct segregation of the mutation within the family (Figure 1b) and to show the absence of the same mutation in 50 healthy controls (not shown). No mutations in HEPC coding region were detected in the remaining 20 patients.

C70R is a missense mutation that changes an aminoacid whose role is pivotal for the final conformation of the protein. All hepcidin cysteines are highly conserved among the different species\textsuperscript{4}, indicating that their role is essential for the function of the mature protein. Also, the C70R mutation is predicted to disrupt the mature peptide, because a neutral aminoacid is substituted by the basic arginine. According to the NMR and structural analysis\textsuperscript{14}, the substituted arginine should disrupt the disulphur bond between the third and the sixth cysteine (Figure 1c) of the peptide. Taken together,
these observations support the causal role of C70R mutation in the molecular pathogenesis of the disease.

C70R is the first missense mutation identified at the homozygous state in HEPC. It is of interest that an amino acid change (G71D), reported to cause hemochromatosis when associated with heterozygous C282Y\textsuperscript{22,23}, occurs adjacent to position 70. G71D occurs independently in patients of different origin\textsuperscript{22,23}. The digenic inheritance of mutations in HEPC and HFE (both G71D and C282Y at the heterozygous state) in iron loaded patients suggests that reduced expression of hepcidin and HFE proteins synergizes in the effect on iron homeostasis. Heterozygous carriers of the proband family, as well as heterozygous carriers of the previously reported HEPC mutations\textsuperscript{3} have normal iron parameters. However, no one had coinherited C282Y at the heterozygous state.

Our results indicate that HEPC mutations leading to JH are rare. Several lines of evidence indicate that hepcidin is a key regulator of iron homeostasis, but its mode of action is little understood. In this context, the identification of HEPC mutations is not only a new diagnostic tool for JH, but may also provide insights into the relationship between the structure of its cognate peptide and its function.
Figure 1. Molecular and biological data of C70R mutation.

a) Electrophoretograms of the HEPC genomic sequence spanning the C70R mutation. The sequence is shown for DNA of a wild type subject (WT), proband (P) and the heterozygous mother (M). The mutation is indicated by the asterisk. 
b) Restriction analysis of C70R mutation by Sac II in the proband family. MWM: molecular weight marker; F = father, P = proband, S1 and S2 = siblings; U: undigest fragment. 
c) Comparison of wild type and mutated peptide aminoacid sequence. The eight cysteines are indicated in green, the mutated arginine in red. 
d) Tridimensional structure of HEPC 25 and 20 aminoacid peptides (from http://www.ncbi.nlm.nih.gov/). The mutated cysteine is indicated in yellow; a white bracket marks the S-S bond disrupted by the mutation.
Table 1. Sequences of the HEPC primers used in the mutation screening.

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>PCR product Length (bp)</th>
</tr>
</thead>
<tbody>
<tr>
<td>exon1 F</td>
<td>5’CAAGCTCAAGACCCAGCAGT3’</td>
<td>206</td>
</tr>
<tr>
<td>exon1 R</td>
<td>5’CAGTGCCCTAGGCTGA3’</td>
<td></td>
</tr>
<tr>
<td>exon2 F</td>
<td>5’CAGTCTCAGAGGTCCACT3’</td>
<td>192</td>
</tr>
<tr>
<td>exon2 R</td>
<td>5’AATGTGAGCAGGGGAACC3’</td>
<td></td>
</tr>
<tr>
<td>exon3 F</td>
<td>5’CAGTGATGCCTTTCTAGC3’</td>
<td>352</td>
</tr>
<tr>
<td>exon3 R</td>
<td>5’AAGGCAGGGTCAGGACAA3’</td>
<td></td>
</tr>
</tbody>
</table>
REFERENCES


Screening hepcidin for mutations in juvenile hemochromatosis: identification of a new mutation (C70r)

Antonella Roetto, Filomena Daraio, Paolo Porporato, Roberta Caruso, Timothy M Cox, Mario Cazzola, Paolo Gasparini, Alberto Piperno and Clara Camaschella