"Screening hepcidin for mutations in juvenile hemochromatosis: identification of a new mutation (C70R)."

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Abstract
Juvenile or type 2 hemochromatosis (JH) is a genetic disease caused by 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 a hepatic antimicrobial-like peptide whose role in iron homeostasis was first defined in animal models; deficiency of hepcidin in mice leads to iron overload, whereas its hepatic overexpression in transgenic animals causes iron deficiency. To define the prevalence of HEPC mutations in JH we screened the HEPC gene for mutation in 21 unrelated JH subjects. We identified a new mutation (C70R), which affects 1 of the 8 conserved cysteines that form the disulfide bonds and are critical for the stability of the polypeptide.

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Brief report

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

Juvenile or type 2 hemochromatosis (JH) is a genetic disease caused by 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 a hepatic antimicrobial-like peptide whose role in iron homeostasis was first defined in animal models; deficiency of hepcidin in mice leads to iron overload, whereas its hepatic overexpression in transgenic animals causes iron deficiency. To define the prevalence of HEPC mutations in JH we screened the HEPC gene for mutation in 21 unrelated JH subjects. We identified a new mutation (C70R), which affects 1 of the 8 conserved cysteines that form the disulfide bonds and are critical for the stability of the polypeptide. (Blood. 2004; 103:2407-2409)

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.1

JH is a heterogeneous genetic disorder, related to at least 2 distinct loci. The first maps to chromosome 1q21,2 but the causal gene is still unknown. A rare subset is due to mutations in hepcidin (HAMP or HEPC)3 gene, which maps to chromosome 19q13.4 As in humans, inactivation of HEPC in mice leads to severe iron overload,5 whereas its overexpression in transgenic mice leads to iron deficiency anemia.6 Recently, it has been demonstrated that an inappropriately low expression of HEPC mRNA is constant in hemochromatosis related to the HFE gene, both in humans7,8 and in animal models.9,10 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.5,11 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.4,11 These soluble forms of hepcidin have been isolated from the urine of healthy subjects. The 25– and 20–amino acid peptides represent the major forms, whereas the 22–amino acid peptide is present only at low concentration.4 A striking feature of the active peptides is the numerous cysteines,8 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 4 disulfide bonds, providing a rigid and tight structure to the final peptide11 (Figure 1D). Extensive promoter analysis has revealed the presence of consensus sequences for the transcription factor CCAAT/enhancer binding protein-α (CEBP/α), which confers liver tissue specificity.12 Hepcidin synthesis is increased by iron loading and inflammation and is inhibited by iron deficiency anemia and hypoxia.13,14 Here we report the results of a study aimed at identifying new HEPC mutations. During the investigation of a large series of patients with JH, 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.

Study design

Twenty-one unrelated patients with JH were studied. The clinical features of most cases have been reported previously.15,16 Six new cases were diagnosed using accepted criteria.17 DNA was prepared from peripheral blood, according to standard protocols. HEPC coding sequences (NT_011109) were amplified by

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polymerase chain reaction (PCR). Primers used for the amplification reaction are reported in Table 1.

PCR was performed in a Gene Amp PCR System 2400 (Applied Biosystems, Foster City, CA), using 25 pmol of each primer and 50 ng template DNA, with an average protocol of 32 cycles (denaturation: 94°C 30 seconds; annealing: 56°C 30 seconds; extension: 72°C 45 seconds) and 1 U AmpliTaq DNA polymerase (Roche Applied Science, Indianapolis, IN).

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 was performed in a Gene Amp PCR System 2400 (Applied Biosystems) according to the manufacturer’s protocols.

Restriction endonuclease digestion was carried out using 20 μL exon 3 PCR product and 10 U SacII enzyme (New England Biolabs, Beverly, MA) in a final volume of 30 μL for 2 hours.

### Results and discussion

Most published information on JH families indicates that they have a genetic disorder that maps to chromosome 1q. Homozygous mutations in HEPC gene were identified in a rare subset of JH patients, with a phenotype indistinguishable from the 1q-linked HEPC mutations in screening Table 1. Sequences of the HEPC primers used in the mutation screening

**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>
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| Exon 1 | F: 5’CAGGCTCAAGACCCAGGAGCAGT3‘
R: 5’CAGGCTCAAGACCCAGGAGCAGT3‘ | 206 |
| Exon 2 | F: 5’CGCTTCAAGGAGGCTCAT3‘
R: 5’AAAGGAGGATACACAGCA3‘ | 192 |
| Exon 3 | F: 5’CAGTGATCCCTTCCCTAGC3‘
R: 5’AAAGCCAAGGTCAAGA3‘ | 352 |
References