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)

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Study design

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

DNA isolation was performed using the Invitrogen DNAeasy Blood and Tissue kit (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. Amplification of the coding sequences of hepcidin (HEPC) using specific primers located at conserved regions of the gene (Supplementary Table 1) was performed using standard PCR conditions (25 cycles; annealing temperature of 60°C). Primers were designed to amplify the 8 conserved cysteines and flanking regions of HEPC. The results of the study are consistent with the hypothesis that hepcidin is a key regulator of iron homeostasis. The data presented here provide further evidence for the involvement of hepcidin in JH and support the concept that increased iron absorption is a key factor in the pathogenesis of this disease.
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 products were electrophoresed in an automatic sequencer (373A; 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.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.2 To establish the frequency of HEPC mutations in JH, we sequenced the HEPC gene of patients from 21 families, regardless of their linkage with chromosome 1q, because linkage may be coincidental when the number of family members is small or the information from 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 an 11-year-old boy with high levels of transferrin saturation, serum ferritin, and liver iron content, but without clinical complications. On the basis of the correct segregation of microsatellite alleles of chromosome 1q within the family, the disorder was previously considered 1q linked.19 The identified nucleotide change causes the substitution of the cysteine at position 70 with arginine (C70R). Because the mutation creates a restriction site for SacII enzyme, SacII digestion of HEPC exon 3 was used 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 amino acid whose role is pivotal for the final conformation of the protein. All hepcidin cysteines are highly conserved among the different species,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 amino acid is substituted by the basic arginine. According to the nuclear magnetic resonance (NMR) and structural analysis,14 the substituted arginine should disrupt the disulfide 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,22,23 occurs adjacent to position 70. G71D occurs independently in patients of different origin.22,23 The digenic inheritance of mutations in HEPC and HFE (both G71D and C282Y at the homozygous 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,3 have normal iron parameters. However, no one had coinherit C282Y at the homozygous 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.
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