Phenotypic and functional data confirm causality of the recently identified hemojuvelin p.r176c missense mutation

In the present study, we correlate homozygosity for the very recently identified *HJV* p.R176C substitution with a juvenile hemochromatosis phenotype. We also show that the p.R176C variant fails to up-regulate the hepcidin promoter activity. Altogether, our results definitively show the R176C amino-acid change to be a novel hemojuvelin loss-of-function mutation.

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Juvenile hemochromatosis (JH) differs from typical *HFE*-related hemochromatosis in that it affects both sexes equally, is linked to a faster iron deposition in parenchymal cells, causes clinical symptoms in the second and third decades of life, and, although liver dysfunction is also part of the syndrome, is associated with a more frequent presentation of hypogonadism and cardiomyopathy. In the absence of treatment, JH patients may succumb to heart failure before the age of 30.[1] JH is genetically heterogeneous since it can be associated with mutations in the *HJV* gene, which encodes hemojuvelin (OMIM 608374), and in the *HAMP* gene, which encodes hepcidin (OMIM 606464).[2] Very recently, Aguilar-Martinez and co-workers have reported the combination of the known p.G320V pathogenic mutation together with a newly identified p.R176C substitution in the HJV gene of a 5-year-old girl of European descent. The girl displayed elevated iron indices without presenting clinical manifestations of juvenile hemochromatosis.[3] We simultaneously detected the p.R176C substitution at the homozygous state in a 17-year-old female of French Caucasian ancestry. At diagnosis, the teenager presented with a transferrin saturation of 97% and a serum ferritin concentration above 2,000 µg/L. Clinical manifestations included astheny, arthralgy, hypogonadotrophic hypogonadism and hepatomegaly. A liver biopsy specimen confirmed diagnosis of hemochromatosis and showed micronoduleous cirrhosis. It should be stressed that, consistent with their respective genetic states (*i.e.* heterozygous or negative for the p.R176C substitution), relatives had normal iron indices (Figure 1). On the other hand, the p.R176C amino-acid change was not detected in 256 healthy blood donors from the same geographical area. Written informed consent was obtained from patients and controls before blood samples were taken.

Hepcidin is a 25 amino-acid peptide that is mainly synthesized by hepatocytes and plays a central role in iron homeostasis. Through its ability to bind the ferroportin iron exporter and cause its degradation, hepcidin determines the amount of iron that must be mobilized from macrophages, enterocytes and hepatocytes to meet the body's needs.[4] As expected from the initial observation of reduced hepcidin levels in patients with *HJV* mutations,[2] and subsequently in *HJV* knock-out mice,[5] there is a strong functional relationship between the two gene products involved in JH. While some important aspects still need to be clarified, a major advance towards a better understanding of this relationship was recently made by Babitt and co-workers. Indeed, these authors have demonstrated that HJV is a bone morphogenic protein (BMP) co-receptor that up-regulates hepcidin synthesis at the transcriptional level via the classical BMP cell-signaling pathway. In turn, they have shown that hepcidin induction by the BMP pathway is significantly reduced in

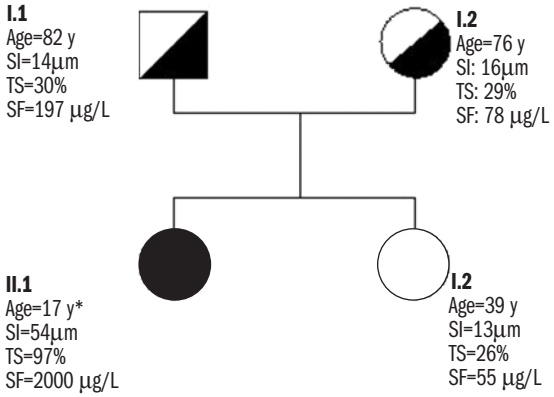
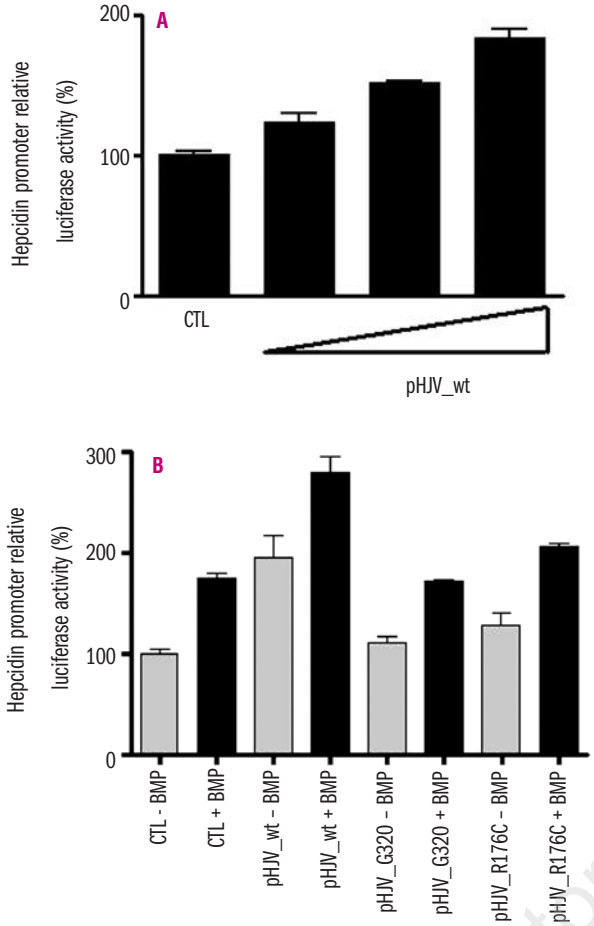


Figure 1. Family Tree. The figure shows the genotype, age, and iron indices (serum iron, transferrin saturation and serum ferritin) for the proband (number II.1) and her relatives. *Data concerning the three relatives were obtained recently, while the proband was diagnosed in 1979. She is now 44 years of age.

hepatocytes lacking HJV. Therefore, they have proposed that enhancement of BMP intracellular signaling by HJV is an important mechanism for regulating hepcidin expression.[6] To demonstrate causality of p.R176C substitution, we designed a hepcidin promoter-based luciferase reporter assay in human Hep3B hepatoma cells and used either HJV or the combination of HJV and exogenous BMP-9 as mediator. BMP-9 was chosen because, at low doses, its potency to activate hepcidin transcription in HepG2 cells had proved to be higher than that of other BMP ligands, and especially BMP-2.[7] We first observed that wild-type HJV increases the hepcidin promoter activity in a dose-dependent manner (Figure 2A) and also confirmed that it enhances the BMP mediated stimulating effect (Figure 2B). We next established that, similarly to the predominant p.G320V HJV mutant, the p.R176C variant fails to stimulate transcription of the hepcidin gene. The failure was apparent with and without BMP-9 addition (Figures 2B), suggesting an important loss of function. HJV exists in a plasma membrane (m-HJV) and a soluble form (s-HJV). Silvestri and co-workers have recently shed new light on the processing of these two forms. In particular. they have shown that m-HJV differs in that it is extensively modified by cleavage of the Asp172-Pro173 bond and the formation of disulfide bonds between the two cleaved protein fragments. They have further demonstrated that cleavage of the Asp172-Pro173 bond, which is supported by a conserved GDPH motif and probably occurs in the late secretory pathway, is necessary for export to plasma membrane. In agreement with these findings, the p.F170S mutant was reported to be efficiently released from Hela cells while it was not cleaved and anchored onto the cell surface as an active form.[8] HJV shares considerable sequence similarity with the Repulsive Guidance Molecules (RGMs) and is the ortholog of a mouse RGM family member (*i.e.* RGMc). Remarkably, orthologs (RGMc) and paralogs (RGMa and RGMb) of human HJV exhibit conserved amino-acid residues surrounding the essential GDPH cleavage sequence. These residues include phenylalanine 170 and arginine 176 (Figure 2C). Considering both the case of the p.F170S mutant and the proposal by Aguilar-Martinez and co-workers of a localized protein structure defect (*i.e.* the



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RGMc_HUMAN      170 FGDPHVRSF
RGMc_MOUSE      163 FGDPHVRSF
RGMc_RAT        165 FGDPHVRSF
RGMa_HUMAN      165 FGDPHLRTF
RGMa_MOUSE      167 FGDPHLRTF
RGMa_CHICK      147 FGDPHLRTF
RGMb_HUMAN      166 FGDPHLRTF
RGMb_MOUSE      169 FGDPHLRTF
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Figure 2. Functional study of the hemojuvelin p.R176C substitution. (A) Hep3B cells, cultured in 6 well plates, were transiently co-transfected with a hepcidin promoter-luciferase reporter plasmid (0.8 μg), a pCMV-βgalactosidase vector (0.2 μg; to control for transfection efficiency) and 0.25 to 1 μg of a pcDNA3.1 plasmid containing the full-length human *HJV* coding sequence (pHJV_wt). As negative control (CTL), cells were incubated with the commercial pcDNA3.1 vector (instead of the pHJV_wt plasmid construct). The hepcidin promoter-based luciferase reporter plasmid construct was generated by cloning the -1458 to +43 region of the human hepcidin promoter (+1 refers to the beginning of transcription; Genbank #AD000684) into the commercial pGL3-basic vector. B. The hepcidin promoter-based luciferase reporter plasmid (0.8 μg) was co-transfected into Hep3B cells with the pCMV-β galactosidase vector (0.2 μg) and either the commercial pcDNA3.1 vector (0.5 μg; CTL) or a pHJV-related plasmid construct (0.5 μg; either pHJV_wt, pHJV_G320V or pHJV_R176C). Twelve hours after transfection cells were serum-starved for 6 hours and treated (+BMP) or not (–BMP) with 0.2 ng/mL BMP-9 for 16 hours. Results are expressed as mean ± s.d., n=3 in each group. They are representative of two independent experiments. Quantitative RT-PCR analysis confirmed that similar amounts of mRNA were generated from all the pHJV plasmid contructs (*data not shown*). (C) Sequence comparisons of the human, mouse, rat and chicken RGM molecules deposited in the Swissprot database (*http://www.ebi.ac.uk/swissprot*). Selected proteins are aligned with the sequence surrounding the GDPH tetra amino-acid motif of human HJV (*i.e.* human RGMc). The residue numbers refer to the putative initiating methionine.

destabilization of a short helix forms by the R176, S177 and F178 residues)[3], it could be suggested that the p.R176C mutant is not efficiently cleaved. It would not, therefore, be able to induce the hepcidin gene transcription from the cell surface. However, as the p.R176C substitution introduces an extra cysteine into a primary amino-acid sequence that naturally comprises 14 cysteine residues, the hypothesis of a critical structural change impairing the HJV folding and its proper trafficking through the ER/Golgi compartments cannot be ruled out. Further experiments are, therefore, required to provide further insight into the biosynthesis and maturation of the p.R176C mutant. In conclusion, our results definitively show the R176C amino-acid change as a novel hemojuvelin loss-of-function mutation.

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Key words: juvenile haemochromatosis, HJV, hemojuveline dysfunction, hepcidin synthesis.

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