Identification of new BMP6 pro-peptide mutations in patients with iron overload

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1 INTRODUCTION

Hereditary Hemochromatosis (HH) is a genetically heterogeneous disorder leading to pathological iron overload (IO), with variable penetrance in terms of timing of onset and the severity of clinical symptoms. The most common HH form is associated with homozygosity for the p.Cys282Tyr mutation in the HFE gene (Type 1 HH), while mutations in at least 4 other genes (HJV, TFR2, SLC40A1, and HAMP) can lead to other HH subtypes collectively designated as “non-HFE” HH. All of the established HH genes are involved in the production and function of the liver hormone hepcidin, the key regulator of systemic iron homeostasis. Notwithstanding the great advances in the molecular diagnosis of HH recently achieved by targeted sequencing, some patients with HH-like phenotypes do not carry mutations in the known genes, suggesting the involvement of still unknown genetic factors. Among others, Bone Morphogenetic Proteins (BMPs) represent compelling candidates. BMPs are cell regulatory proteins belonging to the Transforming growth factor beta (TGF-β) superfamily that play pleiotropic roles in cellular differentiation, proliferation and survival. The BMP/Small Mother Against Decapentaplegic (SMAD) signaling pathway is also critical in iron homeostasis, being involved in transcriptional regulation of hepcidin. Through the interaction with BMP receptors on hepatocyte cell membranes, BMPs can recruit and activate the SMAD 1/5/8-SMAD 4 transcription factor complex, leading to...
TABLE 1  Summary of patients’ data

<table>
<thead>
<tr>
<th>Pt. ID</th>
<th>age/sex</th>
<th>1st level gene test</th>
<th>TS (%)</th>
<th>Ferritin (μg/L)</th>
<th>LIC (μmol/g)</th>
<th>Hepc25 (nM)</th>
<th>Hepc25 ref range (nM)</th>
<th>BMP6 mutation</th>
<th>Reference for variant-IO association</th>
<th>Cofactors</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>#01</td>
<td>54/M</td>
<td>H63D (+/-)</td>
<td>95, 1901</td>
<td>100</td>
<td>&lt;0.5</td>
<td>5.3–8.42</td>
<td>L96P (+/-)</td>
<td>Daher R., et al.201616</td>
<td>Metabolic syndrome. Former alcohol consumption up to 48 g/die.</td>
<td>Died shortly after clinical presentation4</td>
<td></td>
</tr>
<tr>
<td>#02</td>
<td>71/M</td>
<td>wt*</td>
<td>70, 763</td>
<td>95</td>
<td>15.3</td>
<td>5.10–8.41</td>
<td>E112Q (+/-)</td>
<td>New</td>
<td>β-thalassemia minor. Former alcohol consumption up to 36 g/die.</td>
<td></td>
<td></td>
</tr>
<tr>
<td>#03</td>
<td>62/M</td>
<td>H63D (+/-)</td>
<td>33, 1175</td>
<td>91</td>
<td>0.7</td>
<td>6.20–9.36</td>
<td>R257H (+/-)</td>
<td>New</td>
<td>Metabolic syndrome</td>
<td></td>
<td></td>
</tr>
<tr>
<td>#04</td>
<td>65/M</td>
<td>wt*</td>
<td>36, 413</td>
<td>130</td>
<td>7.6</td>
<td>6.20–9.36</td>
<td>R257H (+/-)</td>
<td>New</td>
<td>Metabolic syndrome. Former alcohol consumption 24 g/die</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*For both C282Y and H63D HFE variants.
1Liver Iron Content by MRI or SQUID (n.v. <36).
2Age- and sex-adjusted reference range according to a previous population study.25
3Bronze skin pigmentation, diabetes, decompensated liver cirrhosis.

increased hepcidin expression. In animal models, several pieces of evidence suggest a prominent role of BMP6 in the regulation of iron homeostasis. In wild-type mice fed with diets of different iron content, liver BMP6 expression correlates with body iron stores and with Hamp1 transcription. Moreover, BMP6-deficient mice exhibit a phenotype resembling HH, with an inappropriately low level of hepcidin and tissue iron overload, while injection of BMP6 is able to rescue Hamp1 expression.

According to the currently accepted model, BMP6 is produced mainly by nonparenchymal liver cells in response to sensing of iron stores in order to adjust hepcidin production to body needs. In contrast to the observations in BMP6-deficient mice, direct evidence of a human genetic counterpart has remained elusive until last year, when Daher and colleagues first reported three heterozygous mutations in BMP6 in patients with late-onset IO from five unrelated families. All of the mutations involved the BMP6 prodomain, which is known to be crucial for proper BMP6 processing and secretion, and showed a dominant negative effect. This was elegantly explained by showing the intracellular retention of BMP6 heterodimers composed from wild-type and mutated proproteins. Indeed, BMP6 needs to assemble as a dimer to fully exert its extracellular function in the iron-sensing machinery.

In the present study, we describe four IO patients carrying 3 heterozygous missense mutations in the prodomain region of BMP6 gene, two of which are novel. These findings contribute evidence supporting a role of BMP6 mutations as genetic factors predisposing to human IO.

2 METHODS

2.1 Patients

Our recently described Next-Generation Sequencing (NGS)-based test for IO disorders, updated to include the BMP6 gene (see below for details), was applied to 37 probands (mean age 51.5 ±15.0 years, 89% males), with a putative diagnosis of atypical IO due to hyperferritinemia and increased Liver Iron Content (LIC > 36 μmol/g) detected by magnetic resonance imaging (MRI), as well as a negative or nondiagnostic first level genetic test for HH (i.e., neither p.Cys282Tyr homozygosity nor p.Cys282Tyr/p.His63Asp compound heterozygosity in the HFE gene). Four patients out of this series were found to carry a BMP6 heterozygous mutation. Two were unrelated patients directly attending our Regional Referral Centre for Iron Disorders at the Azienda Ospedaliera Universitaria Integrata of Verona (Italy) (http://www.gimerverona.org), while the third was initially followed at the Azienda Ospedaliera Universitaria San Luigi Gonzaga (Orbassano, Turin, Italy) and later referred to our center for genetic analysis, along with his brother. The latter was the only first-degree relative available for biochemical and genetic analysis in the three families. The main clinical and biochemical features of the patients are summarized in Table 1. All of them were Caucasians of Northern Italian descent and had relevant biochemical signs of IO (i.e., serum ferritin levels up to 1901 μg/L without signs of inflammation ascertained by normal levels of C-reactive protein), confirmed by noninvasive measurement of LIC (> 90 μmol/g) by either MRI (in patients attending the Verona Centre) or superconducting quantum interference device (SQUID) liver susceptometry (in patients initially attending the Turin Centre). A first level genetic test for HH showed simple heterozygosity for HFE p.His63Asp in patient #01 and patient #03. Of note, patient #01 was referred to our center in Verona after being admitted to another hospital for acute decompensation of a previously unrecognized liver cirrhosis. He presented with a clinical phenotype resembling overt, end-stage hemochromatosis, i.e., bronze skin pigmentation, diabetes, and ascites. Liver biopsy was not performed because of prolonged clotting times and thrombocytopenia, and the patient died a few months later. His liver disease was likely multifactorial because of concurrent cofactors (Table 1).
2.2 | BMP6 gene analysis

The BMP6 gene (6p24.3) includes seven exons encoding a proprotein of 513 amino acids, which contains an N-terminal signal peptide, a prodomain (pro-BMP6) essential for appropriate folding, and the relatively small (138 amino acids) C-terminal mature peptide. A customized assay was designed to cover the seven coding exons, flanking intronic boundaries, and UTRs of the human BMP6 gene. Genomic DNA was extracted using a Wizard Genomic Purification kit (Promega, Madison, WI). DNA quality was determined on a Nanodrop 1000 spectrophotometer (Thermo Scientific, Waltham, MA), while the concentration was measured using a Qubit dsDNA assay kit with Qubit fluorometer (Life Technologies, Carlsbad, CA). DNA integrity was assessed by gel electrophoresis on an agarose gel (1%). Targeted capture and sequencing of BMP6, along with the five canonical HH genes (HFE, HV, HAMP, TFR2, and SLCO4A1), was performed according to our previously described method. Briefly, capture of genomic regions was performed starting from 225 ng of gDNA using a custom design HaloPlex™ Target Enrichment 1-500 kb Kit (Agilent Technologies, Santa Clara, CA) according to manufacturer instructions. Library quality was determined using the Agilent High Sensitivity DNA kit on the Agilent 2100 bioanalyzer. The quantity was measured using a Qubit dsDNA assay kit with Qubit fluorometer (Life Technologies, Carlsbad, CA) and by qPCR using the KAPA Library Quantification kit (Kapa Biosystems). Libraries were sequenced with the NextSeq 500 High Output Reagent Cartridge v2 (300 cycles) (Illumina, San Diego, CA) on an Illumina NextSeq 500 sequencer (Illumina, San Diego, CA), generating 150-bp paired-end reads at an average target coverage of 1000X for captured regions. Data analysis and variant validation was performed as previously described. Genomic regions containing the variants of interest were amplified by conventional PCR, and the BMP6 mutations were confirmed in all four patients by Sanger sequencing.

2.3 | Sequence alignment and in silico modeling

Pro-BMP6 sequences from different species were retrieved from UNIPROT and aligned using the MUSCLE program for multiple alignments. Conservation analysis and alignment visualization was performed by Jalview software (Supporting Information Figure S1). Homology modeling techniques were employed to build the BMP6 dimer, using the SwissDock server. TGF-β1 (PDB code: 3RJR) was used as a template. The dimer modeling option was chosen. The generated models were evaluated by using the qmean potential. The best one was then energy-minimized by using Chimera software. The AMBER ff14SB tool was used to perform 100 and 1000 steps of steepest descent and conjugate gradients algorithms, respectively. The positions of the variants in the modeled structure of BMP6 were mapped by Chimera, which was also used to generate the images.

2.4 | Hepcidin measurement

Hepcidin analysis was performed using an updated and validated Mass-Spectrometry (MS)-based assay, which was able to dissect the iron bioactive hepcidin-25 from other hepcidin isoforms. We used a liquid chromatography-tandem mass spectrometry (LC-MS/MS) approach. Briefly, hepcidin-25 synthetic standards (the native and the isotopic labeled internal standard), as well as standards for hepcidin-20 and hepcidin-24 isoforms, were purchased from Peptide International (Louisville, USA). An internal standard was added in all samples, and the calibration curve created. Blank serum (deprived of hepcidin by using charcoal treatment) was used as a reference. Samples were treated by solid-phase extraction using Oasis hydrophilic-lipophilic balanced reversed-phase (HLB) cartridges (Waters, Itaia). High performance LC was performed using an X-Terra MS C18 2.5 μm column (Waters, Itaia), and detection was obtained using a Triple Quad LC-MS/MS (Agilent Technologies). The results were evaluated according to previously obtained reference ranges for males and females at different ages (Table 1). For the sake of brevity, the results are not displayed here but are available upon request.

3 | RESULTS

3.1 | BMP6 mutations and in silico modeling

Our NGS-based test detected 3 different heterozygous pro-BMP6 mutations in the probands. Concurrently, no further potentially pathogenic variant was found in the five canonical HH genes (HFE, HV, HAMP, TFR2, and SLCO4A1), with the exception of the already mentioned p.His63Asp on HFE in patients #01 and #03. Of note, one BMP6 mutation (rs200573175, NM_001718.4:c.287T>G, p.Leu96Pro), detected in patient #01, was the same mutation identified by Daher et al. This mutation is located in exon 1 in the pro-peptide domain, is highly conserved (98%) across different species, and has already been demonstrated to be functional. The other two BMP6 mutations were new. The p.Glu112Gln mutation (rs201486498, NM_001718.4:c.334G>A, p.Glu112Gln) is also located in exon 1 in the pro-peptide domain, adjacent to the pathogenic variant reported by Daher et al. (p.Gln113Glu). On the other hand, the second new mutation (rs148916269, NM_001718.4:c.770G>A, p.Arg257His) is localized to exon 2, corresponding to a different cluster. Arg257 is fully conserved across different species (100%), while Gln112 is only 20% conserved (Supporting Information Figure S1). According to a bioinformatics prediction by Polyphen and SIFT prediction, Arg257 is the most likely pathogenic mutation. Of note, the allele frequency of the two novel BMP6 mutations was quite low in the European non-Finnish population (AF = 0.00019 for p.Arg257His and 5.093e-05 for p.Glu112Gln), as evaluated in the Exome Aggregation Consortium (ExAC; http://exac.broadinstitute.org), which represents the most updated catalogue of human exonic variants and an invaluable tool for efficient filtering of candidate disease-causing variants. We also checked for the presence of the three BMP6 variants in 111 Italian Caucasian subjects with normal iron parameters from another cohort at our hospital. We found two carriers of the p.Leu96Pro mutation and one p.Glu112Gln carrier.
(corresponding to an allele frequency of 0.009), while neither the p.Arg257His mutation nor the p.Glu112Gln mutation were detected.

Our in silico modeling of the BMP6 dimer was built by homology, using the published solved structure of TGF-β1 as a template. The predicted conserved secondary structural elements of the prodomains across members of the TGF-β ligands allowed us to predict the putative localization and functional effects of the mutations detected, as illustrated in Figure 1. The Leu96 and Glu112 amino acids are projected to be located in the latency lasso domain, which encircles the fingers of the opposing monomer, while Arg257 is located in the arm domain.

Leu96 is involved in a putative hydrophobic interaction with Leu99, and its replacement with Pro96 is predicted to cause bumps with nearby residues, creating local perturbations at the backbone level (Figure 1A). Glu112 is a negatively charged residue located near the dimerization interface, and its substitution with a neutral Gln112 residue may change the physicochemical (electrostatic) properties of this region, disrupting these interactions (Figure 1B). Our in silico model is consistent with the cytoplasmic aggregation of BMP6 mutants observed by Daher et al. through immunolocalization in transfected cells. Indeed, both p.Leu96Pro and p.Glu112Gln are located in the same amino acid cluster, in a key region clearly involved in the interaction between the two monomers (Figure 1) and particularly conserved across the TGF-β superfamily. The local perturbations created by these mutations likely affect this interaction, possibly leading to the formation of unfolded dimers that are subsequently degraded.

According to our model, Arg257 may belong to a network of ionic interactions, along with two glutamic acid residues (Glu307 and Glu255). The substitution with a histidine residue could change the pH dependency of the region, altering the local stability. This region of the arm domain, enriched in charged amino acids, appears to be a common feature of TGF-β ligands and is reported to be involved in the interaction with extracellular matrix proteins. Thus, its alteration could theoretically affect either assembly/secretion or final localization of BMP6. However, further experimental data would be needed to verify this hypothesis.

3.2 Serum hepcidin

Hepcidin levels were markedly low in patients #01 and #03 (Table 1). Both patients could be considered in principle as double heterozygous since they also carried the p.His63Asp variant in HFE. Patients #02 and #04 showed slightly increased or normal hepcidin levels, respectively, which in both cases appeared inappropriately low for the iron overload.

4 | DISCUSSION

Notwithstanding great advances in human iron pathophysiology, a number of molecular mechanisms remain to be clarified. Progresses in
the elucidation of these molecular pathways could help for diagnosis and treatment of patients affected by iron-related disorders, particularly those with IO in whom no causative mutation in the known HH genes can be identified.\textsuperscript{3–7} NGS is emerging as a useful approach in this setting.\textsuperscript{6,7,34,35} although patients with evidence of iron overload must to be treated to remove excess iron regardless of if a specific genetic defect can be identified. Cellular and animal models have identified BMPs, particularly BMP6,\textsuperscript{10,12,14} as compelling candidates, but until recently, evidence in humans was limited to the possible role of common SNPs in BMP2 and BMP4 as modifiers of classical HFE-related HH.\textsuperscript{26} The scenario changed early last year, when a French group first reported three heterozygous BMP6 mutations (p.Pro95Ser, 1 patient; p.Leu96Pro, 5 patients; and p.Gln113Glu, 2 patients) associated with an HH-like phenotype.\textsuperscript{16} This raised a debate on causality,\textsuperscript{37–40} although another French group reported 3 additional IO patients carrying the p.Leu96Pro mutation,\textsuperscript{37} and 3 additional carriers of p.Gln113Glu were identified in a large series from UK.\textsuperscript{38} All three mutations were localized to the BMP6 pro-peptide domain, and Daher and colleagues also provided functional studies that consistently suggested an altered secretion and dimerization of the mature BMP6 peptide.\textsuperscript{16} Intracellular BMP6 retention prevented appropriate signaling to the iron-sensing machinery that ultimately regulates hepcidin production according to body iron needs.\textsuperscript{10}

Our results have numerous and striking analogies with those obtained by Daher and collaborators. The two new mutations reported here are both localized to the BMP6 pro-peptide domain and have very low allele frequencies. Our in silico models are also consistent with a possibly damaging functional effect. The critical role of the prodomains in the TGF-β superfamily is widely accepted.\textsuperscript{31} All TGF-β ligands are synthesized as precursors, containing a signal peptide, an N-terminal prodomain, and a C-terminal mature domain. BMPs eventually assemble as dimers before secretion, with prodomains having a crucial role in driving the folding and the dimerization process through a non-covalent interaction with the reciprocal mature domain in both homo- and hetero-dimers.\textsuperscript{41} Regarding BMP6, the highly conserved prodomain region spanning from amino acids 96 to 113 appears particularly important for protein processing and secretion,\textsuperscript{17} and mutations in this amino acid cluster (like the new p.Glu112Gln described in this paper) could be associated with intracellular protein retention.\textsuperscript{17} Indeed, the latency lasso region in which these residues are localized appears fundamental for proper folding of the dimer (Figure 1). Notably, at variance with other BMPs, BMP6 is secreted along with the prodomain, which is eventually cleaved in the extracellular matrix by a convertase (PACE4) associated with heparan sulfate proteoglycans.\textsuperscript{52} The BMP6 prodomain by itself appears to have an important role in facilitating a proper interaction with the extracellular matrix and the subsequent cleavage into the functionally mature peptide,\textsuperscript{43} suggesting the potential pathogenicity of other amino acid substitutions not primarily affecting the protein secretion. For example, in our model, the p.Arg257His substitution, which is located in the arm region, could affect either folding or the interaction with extracellular matrix proteins. However, further experimental data (i.e., molecular biology and site-directed mutagenesis experiments) would be needed to verify these hypotheses.

In addition to molecular analogies, the clinical and biochemical features of our patients are also consistent with those observed by Daher and colleagues.\textsuperscript{16} They concluded that BMP6 mutations were sufficient to explain mild to moderate late-onset IO in males characterized by unexplained hyperferritinemia, increased LIC, and variable transferrin saturation levels (normal to high). Moreover, based on their observations in patients with additional risk factors for IO, they postulated a role for BMP6 mutations in aggravating the accumulation of iron when common conditions such as metabolic syndrome\textsuperscript{44} and excess alcohol intake\textsuperscript{45} coexist. Indeed, all of our patients were males aged 50 years and older with hyperferritinemia, increased LIC and variable transferrin saturation, as well as variable clinical expression according to the presence of cofactors (Table 1). For example, the most severely affected in our series (patient #01) came to our observation with a fully expressed, advanced, HH-like phenotype consisting of “brone diabetes,” with skin hyperpigmentation and liver cirrhosis.\textsuperscript{46} Although he declared an alcohol consumption of approximately four drinks per day during some periods in the past, this was deemed unlikely as the unique explanation for the clinical picture. The patient carried the p.Leu96Pro mutation already reported by Daher and colleagues.\textsuperscript{16} They fully studied this specific mutation by site-directed mutagenesis, immunolocalization in transfected cells, immunohistochemistry, and dimerization studies, all consistent with altered secretion, intracellular retention of BMP6, and reduced signaling for hepcidin activation.\textsuperscript{16} They detected several carriers of the p.Leu96Pro mutation in 5 unrelated families. Such carriers had generally clinical and/or biochemical signs of mild to moderate IO. However, the highest expression (i.e., serum ferritin 1430-4000 μg/l, TSAT 70-99%, and LIC 230-250 μmol/g) was found in two individuals with some cofactors (overweight, metabolic syndrome) and in the sole woman in the French series, who concurrently carried the HFE p.His63Asp mutation (as in our patient #01). Haplotype analyses excluded a founder effect for the p.Leu96Pro mutation, being rather consistent with multiple independent mutational events. Altogether, our data further support a role of the recurrent p.Leu96Pro BMP6 mutation as a predisposing genetic cofactor in determining IO, particularly when associated with acquired risk factors and, possibly, with the HFE p.His63Asp. The pathogenic role of this latter common HFE variant is still a matter of debate. While there is no doubt that its effect is negligible when present alone, population data support a contributory role of p.His63Asp when present in compound heterozygosity with the classical p.Cys282Tyr\textsuperscript{47} or in double heterozygosity with other pathogenic mutations,\textsuperscript{6} particularly in patients with acquired cofactors.\textsuperscript{48} Indeed, our two double heterozygous (BMP6/HFE) patients carrying the p.His63Asp variant had the lowest hepcidin levels (Table 1), although other mechanisms could have contributed, i.e., low protein synthesis by advanced cirrhosis\textsuperscript{49,50} in patient #01. Another 4 patients with atypical IO have recently been reported in whom BMP6 variants appeared to modulate the penetrance of HFE mutations (including p.Cys282Tyr and the rare p.Glu277Lys).\textsuperscript{38} Altogether, these observations agree with the hypothesis of an interaction between BMP6 and HFE,
supported by animal models in which HFE deficiency has been shown to impair BMP6 signaling.\textsuperscript{51,52} The other two BMP6 patients in our series had absolute hepcidin levels either normal or only slightly elevated, i.e., in both cases inappropriately low in relation to the concomitant IO, as observed in the French series.\textsuperscript{16} This resembles what has been observed in classical HFE-related HH, where normal or only slightly increased serum hepcidin levels, as well as inappropriately normal iron absorption, were reported despite the presence of excess liver iron.\textsuperscript{53–55} Overall, the hepcidin levels in the heterozygous BMP6 patients were all consistent with the implication of BMP6 pro-peptide mutations in defective iron signaling and an insufficient hepcidin response.

In summary, we report four additional IO patients carrying three BMP6 mutations, two of which are novel. All mutations were localized to the pro-peptide domain, and our modeling supports the hypothesis that the structural and/or electrostatic changes induced by the variants may affect production of the functional BMP6 mature peptide, leading to an inappropriate hepcidin response.

Our data appear to independently confirm BMP6 as a novel gene to be added to the list of mutant genes contributing to determining an IO phenotype in humans. Apart from highly penetrant juvenile HH subtypes (HJV- and HAMP-related) and similar to classical HFE-related HH, clinical expression of BMP6-related IO appears substantially influenced by acquired cofactors. We predict that with the increasing application of NGS-based tests in IO patients, the full molecular and clinical spectra of BMP6 mutations (possibly including homozygosis or compound heterozygosis for different BMP6 variants) will be fully elucidated.

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DISCLOSURE

MD is a stockholder and scientific advisor of Personal Genomics S.L.R., a spin-off company of the University of Verona. All of the other authors have nothing to disclose.

AUTHOR CONTRIBUTIONS

CP analyzed and validated the variants, reviewed literature/databases, and co-wrote the manuscript. DG designed the research, reviewed the study results and genotype/phenotype correlations, and co-wrote/editing the manuscript. SB performed NGS sequencing. AC performed hepcidin measurements and edited the manuscript. GM, MR, FB, MM, MDG, AR, and OO participated in the clinical enrollment/work-up of the patients. LX and MD performed the NGS bioinformatic analyses and edited the manuscript. ES and AG performed the in silico modeling of mutations and edited the manuscript.

REFERENCES


