HEMATOLOGY, TRANSFUSION AND CELL THERAPY

www.htct.com.br

Original article

Investigation of *BMP6* mutations in Brazilian patients with iron overload

Ana Carolina Mourão Toreli [a,*], *Isabella Toni* [a], *Dulcinéia Martins de Albuquerque* [a], *Carolina Lanaro* [a], *Jersey Heitor Maues* [a], *Kleber Yotsumoto Fertrin* [b], *Paula de Melo Campos* [a], *Fernando Ferreira Costa* [a]

[a] *Hematology and Transfusion Medicine Center, University of Campinas, Campinas, SP, Brazil*
[b] *Division of Hematology, School of Medicine, University of Washington, Seattle, WA, USA*

ARTICLE INFO

Article history:
Available online 16 April 2024

Keywords:
Iron overload
Hemochromatosis
BMP6

ABSTRACT

Background: Iron overload (IO) is a complex condition in which clinical, behavioral and genetic factors contribute to the phenotype. In multiethnic and non-Caucasian populations, mutations in *HFE* gene alone cannot explain IO in most of the cases, and additional genetic and environmental factors must be investigated. Bone Morphogenetic Proteins (BMPs) play a central role in iron homeostasis by modulating *HAMP* transcription through the signaling pathway that includes *SMAD* and *HJV*. In this study, we aimed to explore the clinical relevance of *BMP6* mutations in a cohort of Brazilian patients with IO.

Methods: 41 patients with IO were evaluated. Blood samples were collected to analyze BMP6 mutations through New Sequence Generations (NGS). Frequency of variants and mutations were analyzed and correlated with clinical and environmental characteristics.

Results: We identified *BMP6* mutations in three patients with IO. The p.Arg257His mutation was identified in two patients and the p.Leu71Val mutation was identified in one patient. Two of these patients had additional risk factors for IO (*HFE* mutations and diabetes mellitus).

Conclusion: *BMP6* mutations, when combined to other genetic and clinical risk factors, may contribute to IO. Functional studies and THE evaluation of large cohorts are necessary to fully address BMP6 role in IO.

Introduction

Hemochromatosis is a disease characterized by iron overload (IO) caused by a genetic condition in which hepcidin-ferroportin system is affected and the plasma levels of hepcidin is low. The most common cause of hereditary hemochromatosis in

* Corresponding author at: Hematology and Transfusion Medicine Center, University of Campinas/Hemocentro, UNICAMP, Campinas, Carlos Chagas St, 480, Campinas 13083-878, Brazil.
E-mail address: pmcampos@unicamp.br (A.C.M. Toreli).

https://doi.org/10.1016/j.htct.2024.04.001

Caucasians is a C282Y homozygosis mutation in *HFE* gene. Mutations in *HJV, TFR2, SLC40A1*, and *HAMP* genes have been associated to hemochromatosis, although they are less frequent.[1] Nonetheless, people can develop hemochromatosis owing to a variety of conditions that include other gene mutations. Very probably co-morbidities and behavioral factors play an important role in the phenotype of the disease.[2,3]

Bone Morphogenetic Proteins (BMPs) play a central role in iron homeostasis.[4] BMP6 is produced by the liver cells according to iron stores and, through the signaling pathway that includes *SMAD* and *HJV*, can modulate *HAMP* transcription and consequently hepcidin levels.[1] Daher et al. described three heterozygous missense mutations in *BMP6* (p.Pro95Ser, p.Leu96Pro, and p.Gln113Gl) in six patients with iron overload in whom hepcidin levels were low.[5] Piubelli et al. described the *BMP6* p.Arg257His mutation, which is probably pathogenic due to its location in the protein pro-peptide domain, that is necessary to BMP6 processing and secretion.[1] The p.Val394-Met *BMP6* mutation was described by Alvarenga et al. and its clinical significance is still uncertain.[6] In this study, we aimed to further explore *BMP6* mutations in terms of frequency and clinical relevance in a cohort of Brazilian patients with IO and to correlate with additional genetic and clinical features.

Patients and methods

We prospectively evaluated 41 patients (5 females, 36 males, median age at diagnosis 50 years (31–86 yo) with the diagnosis of IO followed at the Hematology and Hemotherapy Center of the University of Campinas, Brazil. IO was defined by hyperferritinemia (>200 ug in females; >300 ug in males) with transferrin saturation>45 % or evidence of IO on T2* magnetic resonance imaging (MRI). Clinical and laboratory characteristics from patients were collected by medical records evaluation.

Peripheral blood samples were obtained and genomic DNA was extracted from leukocytes using the phenol-chloroform technique. DNA was quantified using a Qubit Fluorometer (Thermo Fisher Scientific, Waltham, MA). A total of 50–500 ng of genomic DNA was fragmented to 140–230 bp by sonication, as measured using a Bioanalyzer 2100 instrument (Agilent Technologies, Santa Clara, CA). For the exome capture platform, genomic DNA libraries were constructed with SureSelect Target Enrichment Kit (Agilent Technologies). Enriched libraries were PCR amplified, pooled, and sequenced on a HiSeq 2500 (Illumina, San Diego, CA) using paired-end 2 × 150 –bp configuration with a minimum read depth of the target region was above 100X.

The sequences obtained were aligned to the human reference genome (GRCh37/hg19) using the BWA program[7] and analyzed using Picard (https://broadinstitute.github.io/picard/) and GATK4[8] to identify variants relevant to the clinical specific indication. Reports were issued on quality control, alignment of reads against the reference human genome GRCh37/hg19 and removal of duplicates. Quality control measures included a minimum average coverage of 150 reads at more than 90 % of positions within a target gene with coverage greater than 100X. The identified variants were recorded in a VCF file (Variant Call Format) and annotated with the wANNOVAR and InterVar algorithms.[9,10] Filtering was performed with VarAFT[11] which included the following criteria: non-synonymous variants, all coding and flanking regions adjacent to exons, splicing sites, 5′-UTR (untranslated region). Reading depth >30 and VAF (variant allele frequency) < 50 %. The variants selected for iron metabolism disorder were considered to be those with 0.1 % maximum allele frequency in the population as reported in 1000 genomes[12] gnomAD database,[13] Exome Variant Server (EVS, http: //evs.gs.washington.edu/EVS/) and ABraOM (Arquivo Brasileiro Online de Mutações)[14] were eligible, considering the clinical condition of the patient. Variants were assessed by mutation prediction and conservation programs including SIFT,[15] Polyphen-2,[16] PROVEAN,[17] MutationTaster2.[18] Then, the relevant variants were submitted to clinical interpretation according to the guidelines established by the ACMG[19] and those variants of unknown significance were manually reviewed.

This research was approved by the Institutional Review Board (number 41684915800005404) and has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki). All participants signed the written informed consent.

Results

We identified *BMP6* mutations in three out of 41 patients with IO (7 %). The p.Arg257His was identified in two patients and the p.Leu71Val mutation was identified in one patient. Among the patients with p.Arg257His mutation, the first was a 68-year-old male who carried a compound heterozygous mutation in *HFE* gene (H63D/C282Y) and presented diabetes mellitus. The second patient was a 71-year-old female in whom no *HFE* mutations or environmental risk factors, were identified. She presented a severe liver cirrhosis with a grade 4 hepatic siderosis on liver biopsy without a clear etiology. The third patient was a 64-year-old male who also carried a compound heterozygous mutation in *HFE* gene (H63D/C282Y) and diabetes mellitus. He presented a moderate IO on the MRI. The p.Leu71Val mutation has not been previously described in the literature. The structure of the protein was analyzed using the Phyre[2] software and the *in silico* analyses demonstrated that this seems not to cause a severe distortion of true molecule (Figure 1). Clinical and laboratory characteristics of the patients are shown in Table 1.

Discussion

Iron overload disorders have been described as a complex condition in which a variety of factors can contribute for its severity. The diagnosis of IO is associated not only with genetic, but also with clinical and behavioral factors.[3] In this study, we identified two different mutations in IO patients: a known p.Arg257His mutation, which was a previously described as a pathogenic variant, and the p.Leu71Val mutation, that was not yet described.

Mutations in *BMP6* gene have been increasingly described as an important issue in the context of IO.[4] Borgel et al. showed that mutations in the pro-peptide region of *BMP6* gene may be associated with defective protein secretion and, consequently, deficient hepcidin secretion.[20] The p.Arg257His

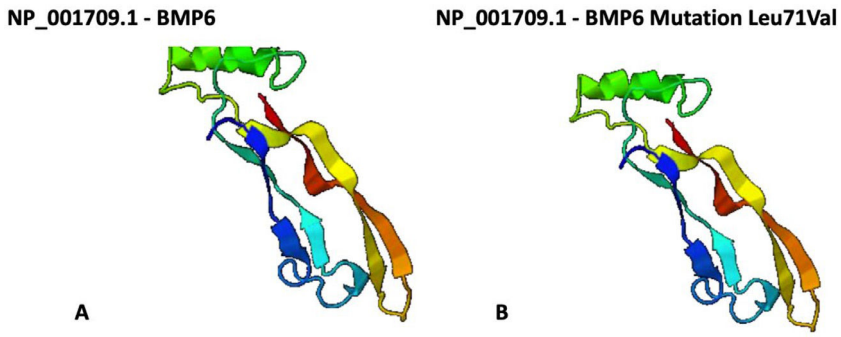

Figure 1 – In silico analysis of BMP6 protein structure. Protein structure did not change when comparing wild-type (A) and p. Leu71Val mutation (B).

Table 1 – Clinical and laboratory characteristics of the patients with IO and *BMP6* mutations.

ID	Age (years)	Sex	*HFE* mutation status	TS (%)	Ferritin (ng/ml)	MRI LIC (mg/g)	*BMP6* mutation	Co-factors
#1	68	Male	C282Y/H63D	71	1490	NA	p.Arg257His Heterozygosis	Type 2 Diabetes
#2	71	Female	Wild type	77	724	NA	p.Arg257His Heterozygosis	Liver cirrhosis
#3	64	Male	C282Y/H63D	41	1982	9,0	p.Leu71Val Heterozygosis	Type 2 Diabetes

MRI LIC, magnetic resonance image – liver iron concentration; TS, transferrin saturation; NA, not available.

mutation was described in two previous studies that evaluated IO. According to these studies, this mutation appears to be pathogenic and related with the clinical phenotype of IO.[1,6] Here we described two patients with the p.Arg257His mutation. The clinical phenotype of patients are in accordance with the characteristics described in the studies by Piubelli et al.[1] and Alvarenga et al.,[6] in which the patients who carried the p.Arg257His mutation presented moderate to severe IO, corroborating the possibility that this mutation is related to the IO phenotype.

In the study described here, the p.Leu71Val mutation, located in the pro-peptide domain of *BMP6* gene, was identified in a male patient with the compound heterozygous HFE mutation C282Y/H63D. Previous data have shown that the C282Y/H63D HFE mutation itself has no significant clinical consequences and cannot explain IO alone.[21,22] To further explore whether this new mutation could be associated with the IO phenotype, we analyzed the protein structure to predict modifications in the molecule and consequently in its function, but no structural mutation was identified. Daher et al. described the p.Leu96Pro mutation associated with IO, which also occurs in the pro-peptide domain, a region important for protein processing and secretion.[5] The mutations are located in the same region, which may indicate the possibility of the association between the p.Leu71Val mutation and IO.

Conclusion

IO is a condition which can be explained by genetic, clinical and behaviors factors. Among genetic features, a mixed pool of mutations with a variety of penetrance could explain different IO grades of severity. Further studies are necessary to identify not only the prevalence of *BMP6* mutations in larger cohorts, but also its influence on the phenotype of the patients with IO. Given the significant ethnic heterogeneity of Brazilian population, the data presented is important to recommend that mutations other than *HFE* should be analyzed in patients with IO of unknown etiology.

Conflicts of interest

The authors declare no conflicts of interest.

Funding

This work was supported by the São Paulo Research Foundation (FAPESP) and the National Council for Scientific and Technological Development (CNPq) – Brazil.

REFERENCES

1. Piubelli C, Castagna A, Marchi G, Rizzi M, Busti F, Badar S, et al. Identification of new BMP6 pro-peptide mutations in patients with iron overload. Am J Hematol. 2017;92(6):562–8.
2. Girelli D, Busti F, Brissot P, Cabantchik I, Muckenthaler MU, Porto G. Hemochromatosis classification: update and recommendations by the BIOIRON society. Blood. 2022;139(20):3018–29.

3. Powell LW, Seckington RC, Deugnier Y. Haemochromatosis. Lancet. 2016;388(10045):706–16.
4. Xiao X, Alfaro-Magallanes VM, Babitt JL. Bone morphogenic proteins in iron homeostasis. Bone. 2020;138:115495.
5. Daher R, Kannengiesser C, Houamel D, Lefebvre T, Bardou-Jacquet E, Ducrot N, et al. Heterozygous mutations in BMP6 propeptide lead to inappropriate hepcidin synthesis and moderate iron overload in humans. Gastroenterology. 2016;150(3):672–83. e4.
6. Alvarenga AM, da Silva NK, Fonseca PFS, Oliveira TGM, da Silva Monteiro JB, Cançado RD, et al. Novel mutations in the bone morphogenetic protein 6 gene in patients with iron overload and non-homozygous genotype for the HFE p.Cys282Tyr mutation. Blood Cells Mol Dis. 2020;84:102444.
7. Li H, Durbin R. Fast and accurate long-read alignment with Burrows-Wheeler transform. Bioinformatics. 2010;26(5):589–95.
8. Heldenbrand JR, Baheti S, Bockol MA, Drucker TM, Hart SN, Hudson ME, et al. Recommendations for performance optimizations when using GATK3.8 and GATK4. BMC Bioinformatics. 2019;20(1):557.
9. Chang X, Wang K. wANNOVAR: annotating genetic variants for personal genomes via the web. J Med Genet. 2012;49(7):433–6.
10. Li Q, InterVar Wang K. Clinical interpretation of genetic variants by the 2015 ACMG-AMP guidelines. Am J Hum Genet. 2017;100(2):267–80.
11. Desvignes JP, Bartoli M, Delague V, Krahn M, Miltgen M, Béroud C, et al. VarAFT: a variant annotation and filtration system for human next generation sequencing data. Nucleic Acids Res. 2018;46(W1):W545–W53.
12. Auton A, Brooks LD, Durbin RM, Garrison EP, Kang HM, Korbel JO, et al. A global reference for human genetic variation. Nature. 2015;526(7571):68–74.
13. Gudmundsson S, Singer-Berk M, Watts NA, Phu W, Goodrich JK, Solomonson M, et al. Variant interpretation using population databases: lessons from gnomAD. Hum Mutat. 2022;43(8):1012–30.
14. DiNardo CD, Cortes JE. Mutations in AML: prognostic and therapeutic implications. Hematology Am Soc Hematol Educ Program. 2016;2016(1):348–55.
15. Ng PC, Henikoff S. SIFT: predicting amino acid changes that affect protein function. Nucleic Acids Res. 2003;31(13):3812–4.
16. Adzhubei IA, Schmidt S, Peshkin L, Ramensky VE, Gerasimova A, Bork P, et al. A method and server for predicting damaging missense mutations. Nat Methods. 2010;7(4):248–9.
17. Choi Y, Chan AP. PROVEAN web server: a tool to predict the functional effect of amino acid substitutions and indels. Bioinformatics. 2015;31(16):2745–7.
18. Schwarz JM, Cooper DN, Schuelke M, Seelow D. MutationTaster2: mutation prediction for the deep-sequencing age. Nat Methods. 2014;11(4):361–2.
19. Richards S, Aziz N, Bale S, Bick D, Das S, Gastier-Foster J, et al. Standards and guidelines for the interpretation of sequence variants: a joint consensus recommendation of the American College of Medical Genetics and Genomics and the Association for Molecular Pathology. Genet Med. 2015;17(5):405–24.
20. Ravasi G, Pelucchi S, Bertola F, Capelletti MM, Mariani R, Piperno A. Identification of novel mutations by targeted NGS panel in patients with hyperferritinemia. Genes. 2021;12(11).
21. Pilling LC, Tamosauskaite J, Jones G, Wood AR, Jones L, Kuo CL, et al. Common conditions associated with hereditary haemochromatosis genetic variants: cohort study in UK Biobank. BMJ. 2019;364:k5222.
22. Borgel A, Lamoril J, Tchernitchko D. Mutations and polymorphisms associated with iron overload in a series of 91 non-HFE haemochromatosis patients. Clin Res Hepatol Gastroenterol. 2020;44(2):239–41.