Association of hepcidin promoter c.-582 A>G variant and iron overload in thalassemia major

Marco Andreani,[1] Francesca Clementina Radio,[4] Manuela Testi,[1] Carmelilia De Bernardo,[4] Maria Troiano,[1] Silvia Majore,[4] Pierfrancesco Bertucci,[3] Paola Polchi,[2] Renata Rosati,[1] and Paola Grammatico[4]

[1]Laboratory of Immunogenetics and Transplant Biology, IME Foundation, Polyclinic of Tor Vergata, Rome; [2]International Center for Transplantation in Thalassemia and SCA, IME Foundation, Polyclinic of Tor Vergata (PTV), Rome; [3]Department of Laboratory Medicine, Polyclinic of Tor Vergata (PTV), Rome, and [4]Medical Genetics, Exp Medicine Dept, "Sapienza-University of Rome", S. Camillo Hospital, Rome, Italy

ABSTRACT

Hepcidin is a 25-amino acid peptide, derived from cleavage of an 84 amino acid pro-peptide produced predominantly by hepatocytes. This molecule, encoded by the hepcidin antimicrobial peptide (*HAMP*) gene shows structural and functional properties consistent with a role in innate immunity. Moreover, as demonstrated in mice and humans, hepcidin is a major regulator of iron metabolism, and acts by binding to ferroportin and controlling its concentration and trafficking. In this study we investigated the influence that mutations in *HAMP* and/or hemocromatosis (*HFE*) genes might exert on iron metabolism in a group of poly-transfused thalassemic patients in preparation for bone marrow transplantation. Our results showed that the presence of the c.-582 A>G polymorphism (rs10421768) placed in HAMP promoter (HAMP-P) might play a role in iron metabolism, perhaps varying the transcriptional activation that occurs through E-boxes located within the promoter.

Key words: *HAMP*, *HFE*, iron metabolism, liver iron concentration, β-thalassemia.

Citation: Andreani M, Radio FR, Testi M, De Bernardo C, Troiano M, Majore S, Bertucci P, Polchi P, Rosati R, and Grammatico P. Association of hepcidin promoter c.-582 A>G variant and iron overload in thalassemia major. Haematologica 2009;94:1293-1296. doi: 10.3324/haematol.2009.006270

Introduction

Sophisticated mechanisms maintain body iron homeostasis and control uptake of dietary iron and its mobilization from stores, in order to satisfy erythropoietic needs and to recycle previously used iron. Communication between cells that consume iron and cells that acquire and store iron must be strictly regulated to provide adequate iron supply while avoiding the toxic effects of elevated iron stores.[1,2]

Hepcidin, encoded by *HAMP* gene, is a recently discovered 25 amino acid peptide that, in addition to being involved in innate immunity,[3] appears to play a crucial role in iron homeostasis in humans, regulating both iron absorption from the intestine and its recycling by macrophages.[4-7] It has been demonstrated that iron overload and inflammation stimulate hepcidin expression while erythropoietic activity decreases its production. Hepcidin synthesis is also suppressed by anemia and hypoxia.[8-10] The HAMP role in iron metabolism has been described in thalassemic patients.[11-14] Additionally, hepcidin deficiency is the cause of iron overload in most forms of hereditary hemochromatosis.[15-21] In this study we investigated the influence of the p.H63D HFE and of the c.-582 A>G HAMP promoter (HAMP-P) gene variants in a group of 97 β-thalassemic patients in preparation for hematopoietic stem cell (HSC) transplant. We analyzed the iron status in patients with a wild type (WT) profile and compared the data to the iron status of patients with p.H63D HFE and/or the c.-582 A>G HAMP-P variants. Estimation of iron overload was based on liver iron concentration (LIC) and serum ferritin (SF) levels.[22-24]

Design and Methods

Patients

All patient-related procedures were approved by the Ethical Review Board. Ninety-seven patients affected by β-thalassemia, originating from an extremely wide geographical area, mainly from Middle Eastern countries, proposed for HSC transplant, were included in the study, as reported in Table 1. Almost all the patients were massively transfused: 70 patients received more than 50 transfusion units (range 51–550, median 148), while 27 received less than 50 transfusion units (6–48, median 26). Thirty-eight patients had been

Manuscript received on January 21, 2009. Revised version arrived on April 2, 2009. Manuscript accepted on April 3, 2009.
Correspondence: Marco Andreani, Ph.D., Laboratorio di Immunogenetica e Biologia dei Trapianti, Fondazione IME, Policlinico Tor Vergata, Viale Oxford 81 00133 Rome. E-mails: m.andreani@fondazioneime.org/m.testi@fondazioneime.org

submitted to regular transfusion therapy since childhood and to chelation with subcutaneous desferrioxamine 5-6 days per week, 8-12 h per day, while 59 patients received irregular iron chelation therapy. Eighteen patients were hepatitis C virus (HCV) positive and showed a median of 43 UI/L alanine transaminase (ALT) levels (range: 23–218).

Clinical parameters

LIC was measured on liver biopsies in 93 patients by acetylene flame atomization and atomic absorption spectrometry (AAnalyst 800, Perkin-Elmer, Waltham, MA, USA). Samples were exsiccated at 120°C for 30' and then submitted to mineralization with nitric and sulphuric acids (100 mL each) at 120°C for 1 h. Finally, samples were diluted to 10 mL with a solution of double distilled water with 1% nitric acid and then analyzed by atomic absorption spectrometry. Liver biopsies were stored at –20°C until analysis. LIC was expressed as mg/g dry weight. Only liver samples with a dry weight of at least 1 mg were considered for the present study. [22,24] Mean value of dry weight liver biopsies was 2.34 mg (range: 1.06-4.58 mg). SF levels were determined by standard methods during routine blood testing prior to transplant on all the 97 patients.

Molecular analysis

A panel of known *HFE* gene mutations (*C282Y, H63D, S65C, Q127H, V53M, V59M, H63H, P160delC, E168Q, E168X, W169X, Q283P*) was screened. Specific regions of the HFE gene were amplified by standard multiplex-PCR and reverse dot-blot was performed according to the kit protocol (Haemochromatosis StripAssay A™-Vienna Lab). HAMP coding regions, intron-exon junctions, and promoter region were analyzed by direct sequence analysis. Primer sequences and PCR conditions for molecular testing are available on request.

Statistical analysis

Statistical analysis was performed on MedCalc Software, (Mariakerke, Belgium) using non-parametric tests including Student's *t* test and Welch's test. Correlations between variables were evaluated by Spearman's correlation coefficient associated with *t* test. A *p* value less than 0.05 was considered statistically significant.

Results and Discussion

The aim of this study was to verify whether HAMP and HFE gene variants affect iron metabolism in polytransfused thalassemic patients as measured by LIC values and serum ferritin levels. These two parameters are reported in the literature to adequately reflect iron load,[22,24] and in our survey reveal a strong correlation, as defined by the Spearman's test. In fact, calculation of the coefficient of determination (R_2) showed that LIC correlated with SF (R_2=0.417, p<0.001). As reported in Table 1, 47/97 patients showed a WT genetic profile in both genes, 25/97 had a variation in HAMP-P, 16/97 in HFE, and 9/97 in both. The classical HFE mutation p.C282Y was not identified in any of the examined patients. All the subjects with an HFE variation were carriers of the p.H63D variant; 24 in the heterozygous and one in the homozygous condition. Regarding the *HAMP* gene, 34/97 patients showed the presence of a heterozygous A/G nucleotide substitution in position -582 where the promoter E-box 1 is located. One out of 97 was a homozygous carrier of the same HAMP-P variant. This transition has been already recognized as a polymorphism affecting a conserved non-coding sequence. The HAMP-P SNP is reported in the dbSNP database (*http://www.ncbi.nlm.nih.gov/projects/SNP/*) with the identification number rs10421768.

A statistically significant difference was observed in the LIC values and SF levels in the group of patients with the c.-582 A>G HAMP-P polymorphism compared to WT patients. As reported in Table 2, the mean LIC value in patients with this mutation was 23.2±12.8 mg/g *vs.* 14.3±9.3 mg/g observed in HAMP-P WT patients (p=0.003), and the mean SF level was 3191±1869 ng/mL *vs.* 2263±1373 ng/mL, in patients with the polymorphism *vs.* controls, respectively (p=0.034). Nine patients with polymorphisms in both *HAMP-P* and *HFE* genes showed a difference in iron overload parameters compared to WT patients, but this difference was not statistically significant, likely due to the small sample size of patients carrying double variants. No statistically significant differences were observed between the groups of patients with or without the HFE p.H63D mutation in mean LIC (15.6±9.8 *vs.* 14.3±9.3 mg/g) or SF (2610±2352 *vs.* 2263±1373 ng/mL) as reported in Table 2.

In the group of 59 patients who received irregular iron chelation therapy, the LIC values and the SF levels were

Table 1. Patients' characteristics.

Patients	97
Median age (years)	9.7, range: 6-28.3
Transfusion treatment	
Less than 50 U	27, range: 6-48, median: 26
More than 50 U	70, range: 51-550, median: 148
Sex	
Female	45
Male	52
Iron chelation treatment	
Regular	38
Irregular	59
Distribution of the different gene mutations	
Wild type	47
Hepcidin promoter c.-582A>G variant	25
HFE p.H63D variant	16
Both mutations	9
Hepcidin promoter c.-582A>G variant	
Homozygous	1 (0.4%)
Heterozygous	24 (96.0%)
Genotype H63D	
H/H	72 (74.2%)
H/D	24 (24.7%)
D/D	1 (1.0%)
HCV positive	18
HCV negative	79

Table 2. Liver iron concentration values (LIC) and serum ferritin levels (SF) in wild type patients (WT), patients with hemochromathosis gene variant (*HFE* p.H63D), and patients with hepcidin gene promoter variant (c.-582A>G *HAMP-P*).

Patients studied	LIC** (mg/g dry weight)	SF (ng/mL)
All	17.1±11.5	2599±1771
n=97*	1.7-47.8	209-9252
WT***	14.3±9.3	2263±1373
n=47	1.7-40.1	491-6376
HFE p.H63D variant	15.6±9.8	2610±2352
n=16	3.5-36.5	209-9252
	NS	NS
c.-582A>G HAMP-P variant	23.2±12.8	3191±1869
n=25	3.3-47.8	443-7590
	p=0.0032	p=0.034

**For measurement of LIC values, n=93 (liver samples with a dry weight less than 1mg were not considered). **LIC (liver iron concentration) normal range is 0.2-1.6 mg/g dry weight. ***statistical analyses are all related to the WT group.*

22.1±11 mg/g and 3299±1853 ng/mL, respectively. When the impact of the c.-582 A>G HAMP-P polymorphism was studied, we observed that the mean LIC and SF levels were significantly higher in the 18 patients carrying the mutation, compared to the 26 WT patients (LIC: 29.7±12 *vs.* 18.2±9.9 mg/g; p=0.0021; SF: 3880±1716 *vs.* 2912±1464 ng/mL; p=0.05). SF levels were higher, but not significantly different, in patients carrying the *HFE* gene mutation compared to the WT group (3455±2814 *vs.* 2912±1464 ng/mL), while the LIC values were comparable (18.7±8 *vs.* 18.2±9.9 mg/g). Among the group of 38 regularly treated patients, LIC and SF values were equivalent in the 3 groups of patients characterized by the different genetic profiles.

Although no functional studies to assess the role of the described HAMP polymorphism on hepcidin expression have been performed, our findings suggest that the presence of this polymorphic variant might play a role in iron metabolism of poly-transfused thalassemic patients,[11-14] perhaps changing the response to the transcriptional activation by both upstream stimulatory factors 1 and 2 (USF1/USF2) and cMyc/Max heterodimers that occur through E-boxes within the promoter, as shown by Bayele.[5] Alterations of these elements might render the promoter less responsive to USF1/USF2 or c-Myc/Max, modifying regulation of the hepcidin transcription factors and its function in iron metabolism.[5]

The c.-582 A>G HAMP-P variant did not show an impact on the level of iron loading in the regular chelated patients, while it influenced the LIC values and the serum ferritin among the patients receiving an irregular chelation treatment. These observations suggest that this HAMP-P polymorphism may be associated with different functionality of the HAMP promoter. The c.-582 A>G HAMP-P substitution might, therefore, represent a risk factor for thalassemic patients by promoting iron overload in these subjects. On the other hand our data showed that this effect can be overcome by a regular and appropriate chelation therapy. Other factors, such as age and variable duration of transfusion regimens, might account for the wide range of LIC and SF levels observed in both WT and HAMP-P variant patients. The reason why in some HAMP-P individuals iron overload was comparable to that of wild-type patients could be due to the fact that they are heterozygous for the SNP and, therefore, show an incomplete penetrance or compensatory mechanisms.

Although it is well known that the presence of the p.C282Y HFE mutation may significantly alter iron absorption regulation,[15-21] this is not always the case for the p.H63D mutation. This common variant is only occasionally associated with iron overload, usually in the homozygous state or in compound heterozygote individuals that also harbor the p.C282Y mutation. Thus, it is not unexpected that our data confirm the results of previous studies showing that the p.H63D heterozygous state does not modify iron loading in thalassemic patients.[19]

In conclusion, although many different factors might be involved, our observations suggest a hypothetical role of the HAMP-P -582 A>G polymorphism on iron metabolism. Investigation into this could be useful as part of the diagnostic and prognostic evaluation of β-thalassemia.

Authorship and Disclosures

MA, MT and PG designed and wrote the paper; MTr organized blood sample collection and made the statistical analysis; FCR performed experiments and reviewed the paper; PB performed the LIC analysis; CDB, PP, RR and SM revised the paper.

The authors reported no potential conflicts of interest.

References

1. Andrews NC. Forging a field: the golden age of iron biology. Blood 2008;112:219-30.
2. Piperno A. Classification and diagnosis of iron overload. Haematologica 1998; 83:447-55.
3. Ganz T. Hepcidin--a peptide hormone at the interface of innate immunity and iron metabolism. Curr Top Microbiol Immunol 2006;306: 183-98.
4. Kemna EH, Tjalsma H, Willems HL, Swinkels DW. Hepcidin: from discovery to differential diagnosis. Haematologica 2008;93:90-7.
5. Bayele HK, McArdle H, Srai SK. Cis and trans regulation of hepcidin expression by upstream stimulatory factor. Blood 2006;108:4237-45.
6. Ganz T. Hepcidin and its role in regulating systemic iron metabolism. Hematology Am Soc Hematol Educ Program 2006;507:29-35.
7. Nemeth E, Valore EV, Territo M, Schiller G, Lichtenstein A, Ganz T. Hepcidin, a putative mediator of anemia of inflammation, is a type II acute-phase protein. Blood 2003; 101:2461-3.
8. Nicolas G, Chauvet C, Viatte L,

Danan JL, Bigard X, Devaux I, et al. The gene encoding the iron regulatory peptide hepcidin is regulated by anemia, hypoxia, and inflammation. J Clin Invest 2002;110:1037-44.
9. Weinstein DA, Roy CN, Fleming MD, Loda MF, Wolfsdorf JI, Andrews NC. Inappropriate expression of hepcidin is associated with iron refractory anemia: implications for the anemia of chronic disease. Blood 2002;100:3776-81.
10. Peyssonnaux C, Zinkernagel AS, Schuepbach RA, Rankin E, Vaulont S, Haase VH, et al. Regulation of iron homeostasis by the hypoxia-inducible transcription factors (HIFs). J Clin Invest 2007;117:1926-32.
11. Origa R, Galanello R, Ganz T, Giagu N, Maccioni L, Faa G, et al. Liver iron concentrations and urinary hepcidin in β-thalassemia. Haematologica 2007;92:583-8.
12. Kattamis A, Papassotiriou I, Palaiologou D, Apostolakou F, Galani A, Ladis V, et al. The effects of erythropoetic activity and iron burden on hepcidin expression in patients with thalassemia major. Haematologica 2006;91:809-12.
13. Camberlein E, Zanninelli G, Détivaud L, Lizzi AR, Sorrentino F, Vacquer S, et al. Anemia in β-thalassemia patients targets hepatic hepcidin transcript levels independently of iron metabolism genes controlling hepcidin expression. Haematologica 2008;93:111-5.
14. Gardenghi S, Marongiu MF, Ramos P, Guy E, Breda L, Chadburn A, et al. Ineffective erythropoiesis in β-thalassemia is characterized by increased iron absorption mediated by down-regulation of hepcidin and up-regulation of ferroportin. Blood 2007;109:5027-35.
15. Kearney SL, Nemeth E, Neufeld EJ, Thapa D, Ganz T, Weinstein DA, Cunningham MJ. Urinary hepcidin in congenital chronic anemias. Pediatr Blood Cancer 2007;48:57-63.
16. Hanson EH, Imperatore G, Burke W. HFE gene and hereditary hemochromatosis: a HuGE review. Human Genome Epidemiology. Am J Epidemiol 2001;154:193-206.
17. Melis MA, Cau M, Deidda F, Barella S, Cao A, Galanello R. H63D mutation in the HFE gene increases iron overload in β-thalassemia carriers. Haematologica 2002;87:242-5.
18. Pietrangelo A, Trautwein C. Mechanisms of disease: the role of hepcidin in iron homeostasis--implications for hemochromatosis and other disorders. Nat Clin Pract Gastroenterol Hepatol 2004;1:39-45.
19. Yamsri S, Sanchaisuriya K, Fucharoen S, Fucharoen G, Jetsrisuparb A, Wiangnon S, et al. H63D mutation of the hemochromatosis gene and serum ferritin levels in Thai thalassemia carriers. Acta Haematol 2007;118:99-105.
20. Le Gac G, Férec C. The molecular genetics of haemochromatosis. Eur J Hum Genet 2005;13:1172-85.
21. Politou M, Kalotychou V, Pissia M, Rombos Y, Sakellaropoulos N, Papanikolaou G. The impact of the mutations of the HFE gene and of the SLC11A3 gene on iron overload in Greek thalassemia intermedia and β(s)/β(thal) anemia patients. Haematologica 2004;89:490-2.
22. Angelucci E, Brittenham GM, McLaren CE, Ripalti M, Baronciani D, Giardini C, et al. Hepatic iron concentration and total body iron stores in thalassemia major. N Engl J Med 2000;343:327-31.
23. Brittenham GM, Cohen AR, McLaren CE, Martin MB, Griffith PM, Nienhuis AW, et al. Hepatic iron stores and plasma ferritin concentration in patients with sickle cell anemia and thalassemia major. Am J Hematol 1993;42:81-5.
24. Angelucci E, Baronciani D, Lucarelli G, Baldassarri M, Galimberti M, Giardini C, et al. Needle liver biopsy in thalassaemia: analyses of diagnostic accuracy and safety in 1184 consecutive biopsies. Br J Haematol 1995;89:757-61.