Brief report

TMPRSS6 rs855791 modulates hepcidin transcription in vitro and serum hepcidin levels in normal individuals

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The iron hormone hepcidin is inhibited by matriptase-2 (MT2), a liver serine protease encoded by the *TMPRSS6* gene. Cleaving the bone morphogenetic protein (BMP) coreceptor hemojuvelin (HJV), MT2 impairs the BMP/son of mothers against decapentaplegic homologs (SMAD) signaling pathway, down-regulates hepcidin, and facilitates iron absorption. *TMPRSS6* inactivation causes iron-deficiency anemia refractory to iron administration both in humans and mice. Genome-wide association studies have shown that the *SNP* rs855791, which causes the MT2 V736A amino acid substitution, is associated with variations of serum iron, transferrin saturation, hemoglobin, and erythrocyte traits. In the present study, we show that, in vitro, MT2 736^{A} inhibits hepcidin more efficiently than 736^{V}. Moreover, in a genotyped population, after exclusion of samples with iron deficiency and inflammation, hepcidin, hepcidin/transferrin saturation, and hepcidin/ferritin ratios were significantly lower and iron parameters were consistently higher in homozygotes 736^{A} than in 736^{V}. Our results indicate that rs855791 is a *TMPRSS6* functional variant and strengthen the idea that even a partial inability to modulate hepcidin influences iron parameters and, indirectly, erythropoiesis. (*Blood*. 2011;118(16):4459-4462)

Introduction

Hepcidin is the key regulator of iron homeostasis, controlling surface expression of the iron exporter ferroportin on enterocytes and macrophages.[1] Inactivation of hepcidin causes severe iron overload in mice and humans, whereas hepcidin overexpression causes iron-deficiency anemia.[2] Hepcidin expression is up-regulated in response to increased body iron, through the bone morphogenetic protein (BMP)–hemojuvelin (HJV)–son of mothers against decapentaplegic homologs (SMAD) pathway[3] and inhibited by matriptase-2 (MT2), a type II transmembrane serine protease encoded by the *TMPRSS6* gene[4,5] that in vitro cleaves the BMP coreceptor HJV.[6] In vivo, "Mask" mice, which have a deleted serine protease domain,[4] and *Tmprss6*-null mice[7] show microcytic anemia because of high hepcidin levels. *TMPRSS6* deleterious mutations in humans cause iron-refractory iron-deficiency anemia that is unresponsive to oral iron administration.[5] The same mutations show partial inhibition of the hepcidin promoter activity when overexpressed with HJV in vitro in hepatoma cells.[6,8]

Recent genome-wide association studies reported the association of common genetic variants of *TMPRSS6* (rs855791 and rs4820268) with serum iron and transferrin saturation,[9-11] hemoglobin, mean corpuscular volume, and mean corpuscular hemoglobin,[12,13] highlighting a role for MT2 in the control of iron and erythrocyte parameters. The SNP rs855791 (2321G- > A) causes a nonsynonymous alanine to valine change (A736V) in the catalytic domain, whereas the SNP rs4820268 leads to a synonymous change at 521 and is in linkage disequilibrium with rs855791. Because rs855791 affects the MT2 catalytic domain, a common speculation was that its effects were hepcidin mediated.[9,14] We tested this hypothesis using an in vitro assay based on the luciferase reporter gene driven by the hepcidin promoter, and showed that the $MT2^{736A}$ inhibits hepcidin more efficiently than $MT2^{736V}$. We also demonstrated that this variant affects the hepcidin levels of normal individuals.

Methods

In vitro studies

The in vitro analyses (Western blot, hepcidin promoter activity assay, and hepcidin-binding assay) were reported previously[6] and are detailed in supplemental Methods (available on the *Blood* Web site; see the Supplemental Materials link at the top of the online article). The *TMPRSS6* variant encoding 736A ($MT2^{736A}$) was obtained by mutagenesis of the $MT2^{736V}$ plasmid using the QuikChange site-directed mutagenesis kit (Stratagene).

Human studies

The population of the genetic isolate "Val Borbera" (VB) was described previously.[15] The study was approved by the San Raffaele ethical committee. Serum hepcidin levels were measured by SELDI-TOF-MS[16] and detailed results are available elsewhere.[11]

Statistical analysis

Association of *TMPRSS6* rs855791 was first analyzed in 655 unrelated (pairwise kinship coefficient < 0.0625) individuals selected using the Greffa software program developed by Falchi et al.[17] We included in the final analysis only individuals with hepcidin levels above the lower limit of

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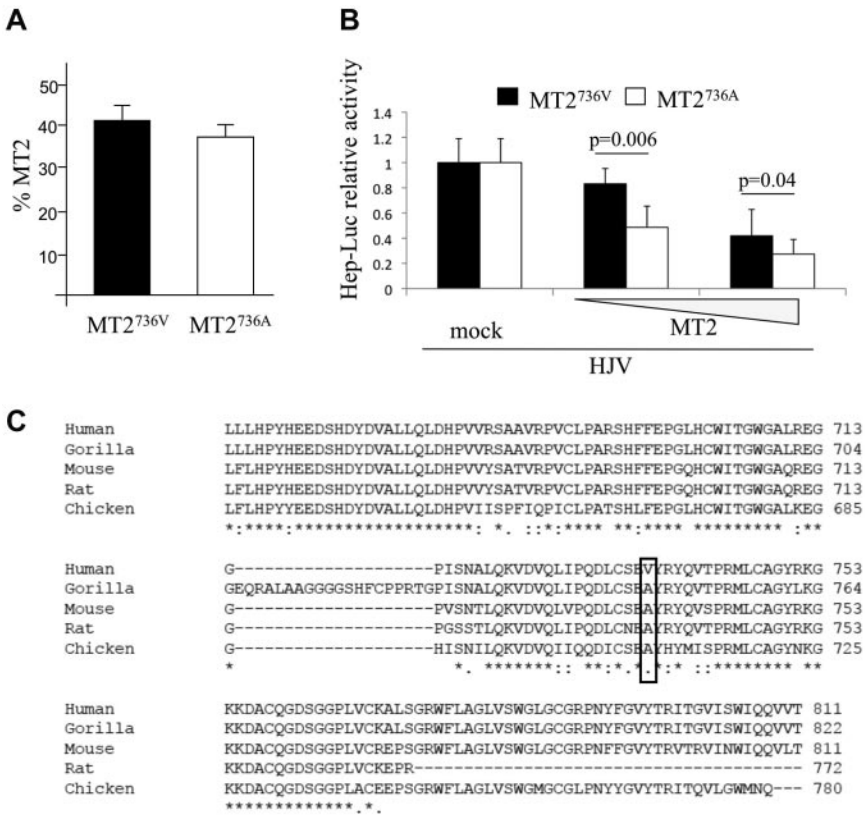

Figure 1. In vitro characterization of the function of MT2 variants and evolutionary conservation of the catalytic domain. (A) Quantification of membrane-bound MT2 (MT2) by binding assay. HeLa cells were transiently transfected with the *TMPRSS6* cDNA encoding MT2^{736V}, MT2^{736A}, or the empty vector (mock) and analyzed for the percentage of MT2 on the cell surface.[6] The amount of surface MT2 was calculated as the ratio between the absorbance of unpermeabilized and permeabilized cells. Error bars indicate SD. (B) Hepcidin promoter activity assay. Hep3B cells were transiently transfected with 0.25 μg of pGL2-basic reporter vector (Promega) containing the 2.9-kb fragment of the human hepcidin promoter[23] in combination with pRL-TK Renilla luciferase vector (Promega) and HJV, as described previously.[6] Increasing doses (from 0.002 to 0.01 μg/mL) of MT2^{736V}- or MT2^{736A}-expressing vectors were used. Relative luciferase activity was calculated as the ratio of firefly (reporter) to Renilla luciferase activity and is expressed as a multiple of the activity of cells transfected with the reporter alone. Experiments were performed in triplicate. The statistical significance is indicated above the bars. (C) Alignment of part of the serine protease domain of MT2 of different species by multiple sequence alignment ClustalW (EMBL-EBI) program. The sequence is highly conserved. The human 736 and the orthologous position in the other species are boxed.

detection (0.55nM) and subjects with ferritin ≥ 30 ng/mL and C-reactive protein ≤ 1mg/dL (subset 1). Mean values were adjusted for sex, age, squared age, and interaction between them (sex*age, sex*squared age) using ANOVA (95% confidence interval) and SPSS 17.0 software and in house R-2.8.1 scripts (The R Project for Statistical Computing at http://www.r-project.org).

Results and discussion

In vitro function of MT2 A736V variants

We first demonstrated that the proportion of MT2^{736A} and MT2^{736V} variants expressed on the surface of transfected cells was similar (Figure 1A). We next observed that the MT2^{736A} variant inhibited the luciferase-hepcidin promoter more efficiently than MT2^{736V}, with a dose-dependent effect at low concentrations (Figure 1B). In agreement with the luciferase assay, the release of the serine protease domain, which we have shown to be correlated with protease activity,[6,8] was slightly increased in cells transfected with the more active MT2^{736A} variant compared with MT2^{736V} (supplemental Figure 1 top panel). These results suggest that rs855791 is a functional variant. Western blot on cell lysates and phospholipase C were not sensitive enough to detect a significant difference in the cleavage of HJV between the 2 variants (supplemental Figure 1 bottom panel).

Based on gene-expression analysis, it was proposed that rs4820268, the other *TMPRSS6* variant that is significantly associated with iron and erythrocyte traits,[10,18] might cause a differential allelic expression (60:40 ratio) of *TMPRSS6* mRNA.[19] However, it is unlikely that the modest difference observed results in detectable changes of the protease activity. rs4820268 is in linkage disequilibrium (R^2 = 0.811 in the VB cohort) with rs855791; therefore, its association with iron and erythrocyte traits might simply be secondary to that of rs855791.

The human database indicates V at the 736 position as the "wild-type" MT2. However, comparative analysis indicates A as the ancestral amino acid, because A is evolutionary conserved in all the species in which an annotated MT2 sequence is available (Figure 1C). This observation suggests that the MT2^{736V} variant, which leads to increased hepcidin production and inhibition of iron absorption, is a recent evolutionary change.

Hepcidin levels of normal carriers of MT2^{736} variants

We validated our in vitro results in the VB cohort, which had serum hepcidin levels measured. Because in a genetic isolate, many individuals are related, only a group of 655 unrelated individuals was studied. We also selected 545 normal subjects after excluding iron-deficient individuals (serum ferritin < 30 ng/mL) and individuals with clinically relevant inflammatory conditions (C-reactive protein > 1 mg/dL).[11] Serum hepcidin levels were lower in AA compared with VV homozygous individuals. The difference was not significant in the whole series, only in the selected subset (P = .038; Figure 2 and supplemental Table 1). Because hepcidin expression is strongly dependent on both iron stores and plasma iron, we normalized hepcidin on ferritin and on transferrin saturation. In both cases, we confirmed that the normalized values were significantly lower in AA compared with VV homozygotes (P = .038 for hepcidin/ferritin and P = .056 for hepcidin/transferrin saturation, respectively) in subset 1 (Figure 2 and supplemental Table 1). Consistently, iron and transferrin saturation were higher in AA than in VV homozygotes (Figure 2 and supplemental Table 1), as was observed previously.[9] Mean corpuscular volume and mean corpuscular hemoglobin showed a similar trend, although

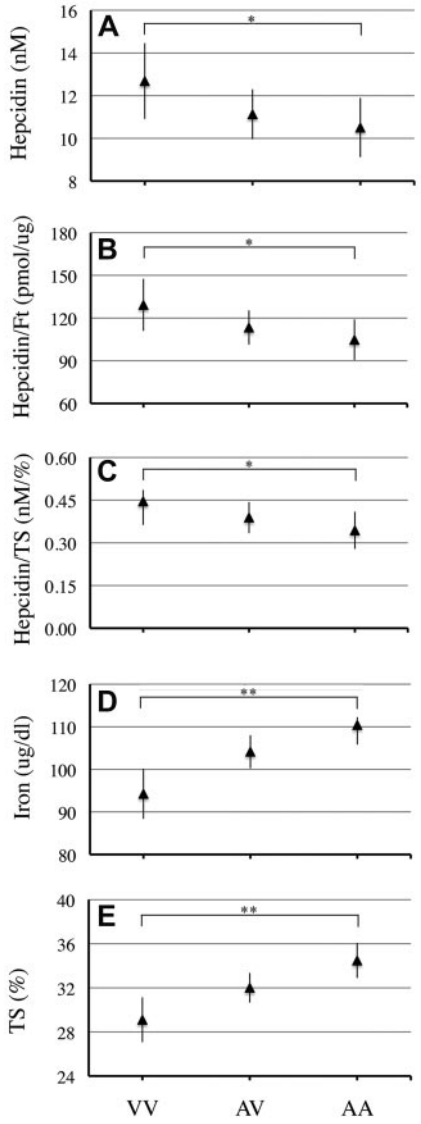

Figure 2. Hepcidin traits and iron parameter mean levels in individuals from subset 1 classified according to MT2 genotypes (AA, AV, and VV). Hepcidin (A), hepcidin/ferritin ratio (B), hepcidin/transferrin saturation ratio (C), serum iron (D), and transferrin saturation (E) are shown. Data are expressed as mean values and are corrected by sex, age, squared age, and interaction by ANOVA (95% confidence intervals are shown). VV indicates homozygotes for the *TMPRSS6* alleles encoding valine; AA, homozygotes for the *TMPRSS6* alleles encoding alanine; and AV, compound heterozygotes for the 2 alleles. *P* values refer to comparison between AA and VV homozygotes; $^{*}P < .05$; $^{**}P < .0005$.

the difference did not reach statistical significance (supplemental Figure 2). No difference was found for ferritin, transferrin, or hemoglobin levels (supplemental Figure 1 and not shown).

Our results suggest that MT2 influences normal hepcidin response to both plasma and total body iron. Hepcidin regulation is complex.[2] In mice, the hepcidin response to isolated increase of transferrin saturation[20] or to an acute iron increase[21] differs from the response to increased total body iron or to chronic iron treatment. Both responses are based on the same BMP-signaling pathway and on SMAD activation, but only the second entails a BMP6 increase.[20,21] The difference in the hepcidin/transferrin saturation and hepcidin/ferritin ratios observed between the two *TMPRSS6* genotypes strengthens a role for MT2 in counterbalancing both BMP6-dependent and BMP6-independent hepcidin up-regulation. The reduced activity of $MT2^{736V}$ demonstrated by the in vitro assay is in agreement with the effect observed in vivo.

$MT2^{736V}$ is the less frequent allele, with a frequency of 0.45 in VB, as in other white populations. From the available studies, the distribution among different populations is not homogeneous (supplemental Table 2). Although the samples analyzed are limited, $MT2^{736A}$ seems largely prevalent among blacks (0.80-0.90) compared with whites (0.50)[9,11,14] and Japanese (0.40).[22] Whether the variant might provide an advantage by enhancing iron absorption in conditions of limited dietary availability or may have conferred protection against certain infections remains to be clarified in future studies.

In conclusion, our data indicate that *TMPRSS6* rs855791 has a functional role in determining protease activity and regulating hepcidin expression both in vitro and in normal subjects, suggesting that it influences the hepcidin response to the increase of both circulating and total body iron.

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Authorship

Contribution: A.N. designed the experimental work, performed the research, and wrote the manuscript; A.P., L.S., N.C., and M.C. performed the research and analyzed the data; M.T. and D.T. performed the statistical analysis and wrote the manuscript; D.G. contributed to the experimental design and wrote the manuscript; and C.C. designed the research and wrote the manuscript.

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References

1. Nemeth E, Tuttle MS, Powelson J, et al. Hepcidin regulates cellular iron efflux by binding to ferroportin and inducing its internalization. *Science.* 2004;306(5704):2090-2093.
2. Hentze MW, Muckenthaler MU, Galy B, Camaschella C. Two to tango: regulation of Mammalian iron metabolism. *Cell.* 2010;142(1):24-38.
3. Babitt JL, Huang FW, Wrighting DM, et al. Bone morphogenetic protein signaling by hemojuvelin regulates hepcidin expression. *Nat Genet.* 2006;38(5):531-539.
4. Du X, She E, Gelbart T, et al. The serine protease TMPRSS6 is required to sense iron deficiency. *Science.* 2008;320(5879):1088-1092.
5. Finberg KE, Heeney MM, Campagna DR, et al. Mutations in TMPRSS6 cause iron-refractory iron deficiency anemia (IRIDA). *Nat Genet.* 2008;40(5):569-571.
6. Silvestri L, Pagani A, Nai A, De Domenico I, Kaplan J, Camaschella C. The serine protease matriptase-2 (TMPRSS6) inhibits hepcidin activation by cleaving membrane hemojuvelin. *Cell Metab.* 2008;8(6):502-511.

7. Folgueras AR, de Lara FM, Pendas AM, et al. Membrane-bound serine protease matriptase-2 (Tmprss6) is an essential regulator of iron homeostasis. *Blood.* 2008;112(6):2539-2545.
8. Silvestri L, Guillem F, Pagani A, et al. Molecular mechanisms of the defective hepcidin inhibition in TMPRSS6 mutations associated with iron-refractory iron deficiency anemia. *Blood.* 2009;113(22):5605-5608.
9. Benyamin B, Ferreira MA, Willemsen G, et al. Common variants in TMPRSS6 are associated with iron status and erythrocyte volume. *Nat Genet.* 2009;41(11):1173-1175.
10. Tanaka T, Roy CN, Yao W, et al. A genome-wide association analysis of serum iron concentrations. *Blood.* 2010;115(1):94-96.
11. Traglia M, Girelli D, Campostrini N, et al. The association of HFE and TMPRSS6 genetic variants to iron and erythrocyte parameters is only in part dependent on serum hepcidin levels. *J Med Genet.* 2011;48(9):629-634.
12. Soranzo N, Sanna S, Wheeler E, et al. Common variants at 10 genomic loci influence hemoglobin A(C) levels via glycemic and nonglycemic pathways. *Diabetes.* 2010;59(12):3229-3239.
13. Oexle K, Ried JS, Hicks AA, et al. Novel association to the proprotein convertase PCSK7 gene locus revealed by analysing soluble transferrin receptor (sTfR) levels. *Hum Mol Genet.* 2011; 20(5):1042-1047.
14. Chambers JC, Zhang W, Li Y, et al. Genome-wide association study identifies variants in TMPRSS6 associated with hemoglobin levels. *Nat Genet.* 2009;41(11):1170-1172.
15. Traglia M, Sala C, Masciullo C, et al. Heritability and demographic analyses in the large isolated population of Val Borbera suggest advantages in mapping complex traits genes. *PLoS One.* 2009; 4(10):e7554.
16. Girelli D, Trombini P, Busti F, et al. A time course of hepcidin response to iron challenge in patients with HFE and TFR2 hemochromatosis. *Haematologica.* 2011;96(4):500-506.
17. Falchi M, Forabosco P, Mocci E, et al. A genomewide search using an original pairwise sampling approach for large genealogies identifies a new locus for total and low-density lipoprotein cholesterol in two genetically differentiated isolates of Sardinia. *Am J Hum Genet.* 2004;75(6):1015-1031.
18. Pichler I, Minelli C, Sanna S, et al. Identification of a common variant in the TFR2 gene implicated in the physiological regulation of serum iron levels. *Hum Mol Genet.* 2011;20(6):1232-1240.
19. Serre D, Gurd S, Ge B, et al. Differential allelic expression in the human genome: a robust approach to identify genetic and epigenetic cis-acting mechanisms regulating gene expression. *PLoS Genet.* 2008;4(2):e1000006.
20. Corradini E, Meynard D, Wu Q, et al. Serum and liver iron differently regulate the bone morphogenetic protein 6 (BMP6)-SMAD signaling pathway in mice. *Hepatology.* 2011;54(1):273-284.
21. Ramos E, Kautz L, Rodriguez R, et al. Evidence for distinct pathways of hepcidin regulation by acute and chronic iron loading in mice. *Hepatology.* 2011;53(4):1333-1341.
22. Kamatani Y, Matsuda K, Okada Y, et al. Genome-wide association study of hematological and biochemical traits in a Japanese population. *Nat Genet.* 2010;42(3):210-215.
23. Pagani A, Silvestri L, Nai A, Camaschella C. Hemojuvelin N-terminal mutants reach the plasma membrane but do not activate the hepcidin response. *Haematologica.* 2008;93(10):1466-1472.