Reduced sensitivity of the ferroportin Q248H mutant to physiological concentrations of hepcidin

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ABSTRACT

Ferroportin Q248H mutation has an allele frequency of 2.2-13.4% in African populations and is associated with a mild tendency to increased serum ferritin in the general population. Some investigators have reported that ferroportin Q248H is degraded after exposure to hepcidin in exactly the same manner as wild-type ferroportin, but supraphysiological concentrations of hepcidin were used. The aim of our study was to determine whether ferroportin Q248H may have reduced sensitivity to physiological concentrations of hepcidin. The sensitivity of ferroportin Q248H to hepcidin was determined in 293T cells transiently expressing ferroportin using immunoblotting and fluorescence analysis. Ferritin concentrations were measured in these cells and also in human primary monocytes derived from humans with different ferroportin genotypes. The effect of Q248H on serum iron measures was examined in patients with sickle cell anemia. Immunoblotting and fluorescence analysis showed decreased sensitivity of ferroportin Q248H to physiological concentrations of hepcidin. Lower ferritin concentrations were observed after incubation with iron and hepcidin in 293T cells expressing ferroportin Q248H and in primary monocytes from ferroportin Q248H subjects. In sickle cell anemia, ferroportin Q248H heterozygotes had lower serum ferritin concentrations than wild-type subjects, consistent with enhanced iron release by macrophage ferroportin Q248H. A clinical benefit of ferroportin Q248H was suggested by lower echocardiographic estimates of pulmonary artery pressure in patients carrying mutant alleles. In conclusion, our results suggest that ferroportin Q248H protein is resistant to physiological concentrations of hepcidin and that this mutation has discernible effects on iron metabolism-related clinical complications of sickle cell anemia. They provide a mechanistic explanation for the effect of ferroportin Q248H on iron status in individuals of African descent and suggest that these changes in iron metabolism may be beneficial under certain disease-specific circumstances. *(ClinicalTrials.gov Identifier:NCT00011648).*

Introduction

Ferroportin is the sole iron export protein to have been identified in mammals and is responsible for iron export from enterocytes of the duodenum, macrophages and other cells.[1-5] In duodenal enterocytes, ferroportin protein is expressed on the basolateral membrane and exports iron into the portal blood circulation. In macrophages, ferroportin is expressed in intracellular vesicles and localizes to the cellular membrane to facilitate iron export.[6-8] Hepcidin is synthesized mainly by hepatocytes[9] and also by macrophages.[10] Binding of hepcidin to ferroportin induces ferroportin internalization and degradation.[9] Removal of ferroportin from the cell surface leads to cellular iron retention and an increase in cellular ferritin, the major iron storage protein. Mutations that lead to decreased production of hepcidin or that reduce the sensitivity of ferroportin to hepcidin lead to higher dietary iron absorption and increased parenchymal cell iron stores.[11] Measured physiological serum hepcidin concentrations vary but typically stay in the low nanomolar ranges of 0.001-0.01 μM in healthy subjects and increase to 0.05-0.1 μM in subjects with high ferritin levels.[12-15]

Ferroportin Q248H (rs11568350; negative strand DNA 744G T substitution) is a common variant limited in distribution to populations of African ancestry with a variant allele frequency ranging from 2.2% to 13.4% depending on the population studied.[16-22] Ferroportin Q248H is associated with a mild increase in serum ferritin concentrations among adults.[17,18,20] However, Q248H was not linked to decreased expression of ferroportin or to changes in ferroportin sensitivity to hepcidin in previous studies that used supraphysiological concentrations of hepcidin.[23] One study found that ferroportin Q248H expressed in *Xenopus* oocytes had reduced ability to export iron, exporting about 25% of that exported by wild-type (WT) ferroportin.[24] In Zimbabwean children,[19] the Q248H variant seemed to protect from iron deficiency, but this effect was predominantly observed in those children who also had an elevated C-reactive protein concentration suggesting an underlying inflammatory process. This observation, along with the finding that children with iron defi-

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ciency or ferroportin Q248H had lower circulating tumor necrosis factor-α concentrations than iron-replete children with WT ferroportin,[25] led us to hypothesize that ferroportin Q248H has reduced sensitivity to hepcidin but that this property would be observed at lower concentrations of hepcidin than those used in previous studies.[23] Here, we analyzed the sensitivity of ferroportin Q248H to hepcidin at various concentrations by determining the levels of ferroportin transiently expressed in cultured cells and in human primary monocytes derived from humans with different ferroportin genotypes. We also measured ferritin concentrations in these cells, levels that are indicative of cellular iron status. Finally, we examined the effect of Q248H on serum iron measures in patients with sickle cell anemia who have markedly increased macrophage iron export because of chronic hemolysis.

Design and Methods

Predictive analysis and worldwide allele frequencies

Minor allele frequencies for missense mutations in the single nucleotide polymorphism database (dbSNP) were determined using Kaviar and sequence data (*http://db.systemsbiology.net/kaviar*).[26] Mutations were analyzed for effects on protein function using a 571-residue ferroportin protein (UniProtKB/Swiss-Prot Q9NP59) and the predictive analysis tools: SIFT (Sorting Intolerant From Tolerant; *http://blocks.fhcrc.org/sift/SIFT.html*)[27] and PolyPhen2 (Polymorphism Phenotyping version 2; using HumDiv model; *http://genetics.bwh.harvard.edu/pph2/index.shtml*).[28]

Cells and media

293T cells were purchased from ATCC (Manassas, VA, USA) and cultured in Dulbecco's modified Eagles medium (DMEM) containing 10% fetal bovine serum (FBS) (Life Technologies) and 1% glutamine (Invitrogen, Carlsbad, CA, USA) at 37°C in the presence of 5% CO_2.

Ferroportin expression constructs

Human WT ferroportin, ferroportin Q248H and ferroportin C326Y mutants cloned with c-Myc and histidine tags in a pcDNA3.1 expression vector were kindly provided by Dr. Hal Drakesmith.[23] To generate enhanced green fluorescent protein (EGFP)-tagged human ferroportin, the ferroportin coding sequences from WT ferroportin, ferroportin Q248H and ferroportin C326Y mutants were amplified by polymerase chain reaction (PCR) with the following primers that included XhoI and Kpn1 restriction sites (shown in italics): forward primer, GC*CTCGAG*ATGACCAGGGCGGGAGATCAC and reverse primer, GC*GGTACC*GTAACAACAGATGTATTTGCTTGATTTTC. The PCR products were purified on agarose gel, digested with *XhoI* and *Kpn1* (BioLabs, Ipswich, MA) and ligated into the pEGFP-N1 vector (Clontech, Mountain View, CA, USA) which was also digested with *XhoI* and *Kpn1* and ligated. The ligation products were transformed into *E. coli* DH5α cells (Invitrogen) and kanamycin-resistant colonies were selected. WT ferroportin-EGFP, C326Y and Q248H ferroportin -GFP-expressing plasmids were purified using a Qiagen (Valencia, CA, USA) purification kit and sequenced using the Macrogen service (Rockville, MD, USA).

Transfection and treatment

293T cells were seeded in 6-well plates to achieve 50% confluence at the day of transfection. The cells were transfected with the indicated plasmids using Lipofectamin and Plus reagents (Invitrogen), following the manufacturer's protocol. The efficiency of transfection was verified by green fluorescence of ferroportin-EGFP or co-expression of EGFP from the CMV-EGFP (Clontech, Mountain View, CA, USA) reporter. For the hepcidin treatment, chemically synthesized hepcidin/LEAP-1 (human) was used (Peptide Institute Inc., Osaka, Japan).

Western blotting

293T cells were washed with chilled phosphate-buffered saline (PBS), and whole-cell lysates were prepared with 1X sodium dodecylsulfate (SDS)-loading buffer (50 mM Tris-HCl pH 6.8, 2% SDS, 10% glycerol, 1% β-mercaptoethanol, 0.1% bromophenol blue), heated at 70°C for 5 min and separated on 8% polyacrylamide gel, transferred to polyvinylidene fluoride membranes (Millipore, Allen, TX, USA). Membranes were blocked for 30 min in 5% non-fat dry milk in PBS (pH 7.4), containing 0.1% Tween-20. The blots were incubated overnight at 4°C with 1:1000 rabbit anti-c-Myc tag (Upstate, Lake Placid, NY, USA) or rabbit anti-GFP (Santa Cruz, CA, USA), then washed with PBS/0.05% Tween-20 and incubated for 30 min with a 1:5000 dilution of anti-rabbit IgG F(ab')2 fragment linked to horseradish peroxidase (GE Healthcare, Piscataway, NJ, USA). As a loading control, tubulin was detected with anti-α-tubulin monoclonal antibodies (Sigma, St. Louis, MO, USA). Immunoblots were developed using the Pierce Super Signal West Pico Chemiluminescent Substrate Kit (Thermo Fisher, Marietta, OH, USA).

Fluorescence measurements of enhanced green fluorescent protein

293T cells were transfected with ferroportin-EGFP expressing vector, treated with hepcidin and then washed with PBS, and resuspended in 100 μL of PBS/well in a 96-well white plate. The fluorescence was measured at 488 nm excitation and 510 nm emission (Perkin-Elmer, Boston, MA, USA).

Preparation of human primary monocytes and macrophages

Peripheral blood mononuclear cells were isolated from 20 mL of whole blood collected in EDTA-containing vacutainer tubes. The Howard University Institutional Review Board committee approved the protocol and the donor signed the consent form. The whole blood was diluted (1:1) with PBS and applied on Ficoll-Hypaque and centrifuged at 400xg for 30 min. The peripheral blood mononuclear cells were removed from the interphase and further washed with PBS. Peripheral blood mononuclear cells were further enriched for monocytes using the Dynabeads monocyte separation kit from Invitrogen following the manufacturer's instructions. In brief, peripheral blood mononuclear cells were mixed with blocking reagent (aggregated gamma globulins in 0.9% NaCl and an antibody mix of mouse IgG for CD3, CD7, CD16, CD19, glycophorin A in PBS and sodium azide) for 20 min at 4°C. Cells were then washed and added to pre-washed magnetic beads for 15 mins at 4°C. Magnets were applied for 3 min and the supernatant containing pure untouched monocytes was transferred to a fresh tube in RPMI 1640. Primary monocytes were then converted to macrophages using 20 ng/mL of granulocyte colony-stimulating factor (Pepro Tech Inc., Rocky Hill, NJ, USA).

Measurement of cellular ferritin

293T cells transfected with ferroportin expression vectors or primary macrophages were incubated with 100 μM ferric ammonium citrate (FAC) for 16 h. The cells were then treated with 100 μM cycloheximide (Sigma) for 1 h followed by incubation for 24 h in the absence or presence of the indicated concentrations of hepcidin. The cells were lysed with ferritin lysis buffer (50 mM Tris-HCl pH 8.0, 150 mM NaCl 5 mM EDTA, and 0.5% NP-40) for 10

min at 4°C and then centrifuged at 10,000x g for 30 min to precipitate nuclear material and organelles. Supernatants were collected and protein concentration was measured using the Bradford assay (Bio-Rad). Total cellular ferritin was measured in the lysates using ferritin enzyme-linked immunosorbent assay (ELISA) kits obtained from Biotech Diagnostic (Laguna Niguel, CA, USA).

Patients with sickle cell disease

Study subjects included 284 adults with sickle cell anemia (hemoglobin SS or hemoglobin $S\beta^0$-thalassemia) and genomic DNA suitable for PCR testing who were screened for pulmonary hypertension on an NHLBI IRB-approved protocol (ClinicalTrials.gov Identifier:NCT00011648) between 2001 and 2007. Details of the clinical and echocardiographic methods and study results for many of these patients have been reported previously.[29-32] Complete blood counts, serum chemistries and measurements of iron status were performed by routine methods. N-terminal pro-brain natriuretic peptide (NT-proBNP) concentrations were determined by a sandwich immunoassay utilizing polyclonal antibodies that recognize epitopes located in the N-terminal segment (1–76) of pro-BNP (1–108) (Elecsys analyzer; Roche Diagnostics, Manheim, Germany). Tricuspid regurgitation velocity (TRV), which reflects estimated systolic pulmonary artery pressure, was determined by echocardiography.[29,33] Among 33 African-American control subjects in our laboratory with a measurable TRV, the median TRV was 2.0 m/sec with a 5th percentile to 95th percentile range of 1.8 to 2.5 m/sec.[29]

Ferroportin Q248H (rs11568350) genotyping

Exon 6 of ferroportin was amplified by PCR from genomic DNA using a set of primers encompassing portions of the introns that flank the exon (forward primer: 5'-CAT CGC CTG TGG CTT TAT TT-3'; reverse primer: 5'-GCT CAC ATC AAG GAA GAG GG-3'). PCR reactions were performed in 25 μL volumes in a standard PCR buffer containing 1.5 mM $MgCl_2$, 200 μM dNTP, 20 nM Primers and 0.5U Taq DNA polymerase. After initial denaturation at 95°C for 5 min, a PCR was performed in a thermocycler (Bio-Rad) for 38 cycles of heating at 95°C for 15 s, annealing at 55°C 15 s and extension at 72°C for 1 min. A final cycle of 10 min at 72°C was also added. Ten microliters of PCR product (392 bp) were digested with *PvuII* enzyme (MBI Fermentas, Hanover, MD, USA) for 2 h at 37°C, and the resulting DNA fragments (252 bp and 140bp) were resolved on 2.5% agarose gel and detected with ethidium bromide staining.

Statistical methods

Clinical measurements in patients with sickle cell anemia were compared according to ferroportin Q248H status in unadjusted analyses. If the TRV was not measurable, the patient was excluded from the TRV analysis. The association of TRV, categorized as values <2.5 m/sec, 2.5-3.0 m/sec, or >3.0 m/sec, with ferroportin Q248H was assessed by the Cochran linear trend test. Patients with sickle cell disease often receive transfusion therapy, and iron stores are strongly influenced by the total number of lifetime blood transfusions received as each unit of blood contains 200-250 mg of iron which are eventually added to stores. To determine the effect of ferroportin Q248H on indirect measures of iron stores, we performed subanalyses on patients reporting having received fewer than five transfusions during their lifetime.

Results

Ferroportin mutations in the same intracellular domain as Q248H

We first reviewed the medical literature to determine the characteristics of ferroportin mutations that flank Q248H and that involve the same predicted intracellular domain of the protein (Table 1). *In silico* predictions suggest that L233P is the only variant in this domain likely to have a dramatic effect upon ferroportin function, although nearly all of these missense mutations are associated with a phenotype in humans (Table 1). Ferroportin Q248H is the only missense change that is a polymorphism with an observed worldwide minor allele frequency >1% in a query of large human DNA sequence databases.

Myc-tagged ferroportin Q248H is resistant to physiological concentrations of hepcidin

Hepcidin binds to ferroportin and induces its internalization and degradation, resulting in cellular iron retention and decreased iron export. First, we examined the effect of hepcidin on ferroportin Q248H expression in human embryonic kidney 293T cells that naturally express undetectable levels of ferroportin.[34] Cells were transiently transfected with plasmids expressing ferroportin Q248H, WT ferroportin or ferroportin C326Y, a mutant that is not sensitive to hepcidin.[34] The EGFP expression plasmid was

Table 1. Ferroportin variants that arise from mutations that flank c.744G→T (Q248H) and result in changes in the same intracellular domain.

Nucleotide change	Missense mutation	Hg19 position	dnSNP	Worldwide MAF (%)	SIFT	Polyphen2	Phenotype	Sensitivity to hepcidin	Surface expression and Fe^{2+} export
c.698T→C	L233P (60, 61)	190430142	Rs186912553	0.04	AFP	Possibly	Increased macrophage and hepatocyte iron	Decreased	Decreased
c.718A→G	K240E (38, 44)	190430122	-	0.0	Tolerated	Benign		Decreased	Increased
c.744G→T	Q248H (16, 62)	190430096	Rs11568350	1.19	Tolerated	Benign	Increased ferritin	Not known	Not known
c.800G→A	G267D (63-66)	190428912	Rs104893664	0.0	Tolerated	Possibly	Increased ferritin	Not known	Not known
c.809A→T	D270V (67, 68)	190428903	-	0.0	Tolerated	Benign	Increased ferritin	Not known	Not known

HumDiv model used with Polyphen2 for interrogation of human polymorphisms. MAF: minor allele frequency.

used as a negative control. Transfected cells were treated with 0.01 μM, 0.03 μM or 0.1 μM hepcidin for 1 h and ferroportin expression was determined by immunoblotting with anti-cMyc antibodies and compared to the loading control with anti-α-tubulin antibodies (Figure1A). While the expression of WT ferroportin was reduced following treatment with 0.01, 0.03 and 0.1 μM hepcidin (Figure 1A, lanes 6, 7 and 8), ferroportin Q248H expression persisted following treatment with 0.01 and 0.03 μM hepcidin (Figure 1A, lanes 2 and 3) and was only reduced by treatment with 0.1 μM hepcidin (Figure 1A, lane 4). In contrast, ferroportin C326Y expression persisted at all hepcidin concentrations with a slight reduction when hepcidin was added (Figure1A, lanes 10-12). Analysis of ferroportin Q248H expression conducted in three independent experiments showed a resistance to 0.03 μM hepcidin and no effect at the higher 0.3 μM hepcidin concentration in comparison to WT ferroportin (Figure1B). Thus, ferroportin Q248H was less sensitive to the low hepcidin concentrations tested.

Expression of ferroportin Q248H reduces cellular ferritin in iron-treated cells

Intracellular ferritin levels reflect the amount of iron stored within cells. Ferroportin Q248H, WT ferroportin or ferroportin C326Y were expressed in 293T cells. Transfected cells were incubated with 100 μM ferric ammonium citrate as a source of iron for 16 h. The cells were then treated with 100 μM cycloheximide for 1 h followed by 18 h incubation with 0.03 μM hepcidin. Ferritin levels increased 2.6-fold in cells expressing ferroportin Q248H that were treated with hepcidin compared to 4.1-fold in cells expressing WT ferroportin that were treated with hepcidin (Figure 1B, P=0.0004). Ferritin levels were the same in untreated and hepcidin-treated cells expressing ferroportin C326Y (Figure 1B). After exposure to 0.03 μM hepcidin, the lower ferritin levels in cells expressing ferroportin Q248H compared to those expressing WT ferroportin suggest continuing iron export by ferroportin Q248H with low dose hepcidin and support the possibility that the Q248H amino acid substitution has mild resistance to hepcidin compared to the fully sensitive WT ferroportin.

Ferroportin Q248H-EGFP mutant is resistant to physiological concentrations of hepcidin

Ferroportin is a membrane-associated protein that is quickly degraded after cell lysis. Thus measurement of ferroportin expression by immunoblotting is difficult and requires semi-denaturing lysis conditions[35] that may not correctly recapitulate ferroportin expression levels. To determine ferroportin expression in a physiological context, we measured fluorescence of an EGFP-fused ferroportin Q248H mutant in live cells. EGFP-tagged human ferroportin Q248H, WT ferroportin, and ferroportin C326Y were generated by subcloning WT and mutant ferroportins into an EGFP-expression vector. EGFP-fused ferroportins were expressed in 293T cells. Cells were treated with 0.01 μM, 0.03 μM, or 0.1 μM hepcidin for 4 h and fluorescence was measured in live cells. Measurement of ferroportin-fused EGFP fluorescence again showed that, compared to WT ferroportin, the Q248H mutant was somewhat resistant to 0.03 μM hepcidin but not 0.1 μM hepcidin (Figure 2A). These results were further confirmed by comparing immunoblots of EGFP-fused ferroportin Q248H and WT ferroportin after exposure to hepcidin (Figure 2B). While 0.03 μM hepcidin reduced WT expression but not Q248H mutant ferroportin (Figure 2B, lanes 2 and 5), at 0.3 μM hepcidin expression of both WT and Q248H ferroportins was reduced (Figure 2B, lanes 3 and 6).

Effect of hepcidin on ferroportin Q248H in human primary macrophages

We next examined ferritin levels in primary macrophages differentiated from monocytes obtained from patients with homozygous Q248H, heterozygous ferroportin Q248H or homozygous WT ferroportin (Figure 3). Macrophages were treated with ferric ammonium citrate for 18 h and then treated with 0.01-0.5 μM hepcidin for 6 h. While treatment of macrophages with WT ferroportin increased the ferritin level by 2.5-fold, ferritin was only increased 1.5-fold in

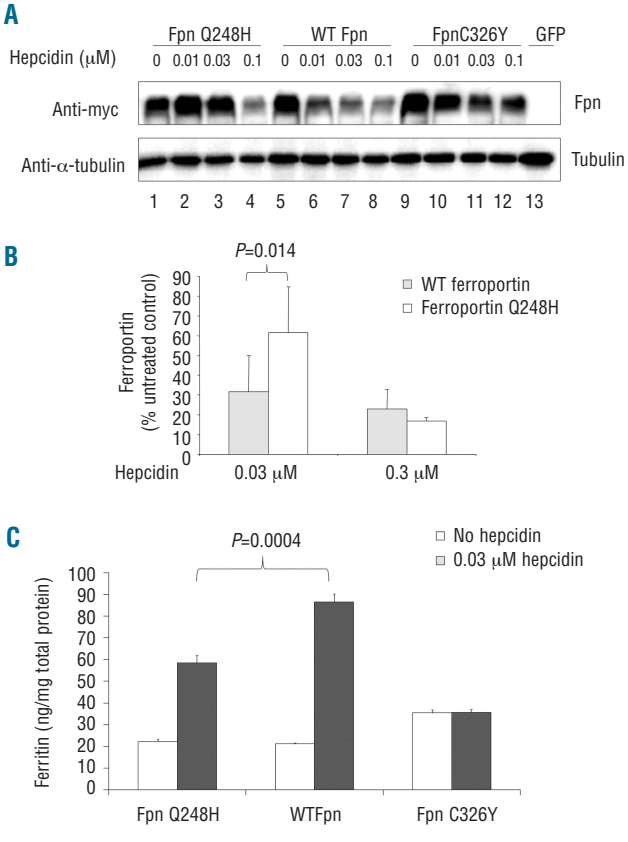

Figure 1. Ferroportin Q248H is less sensitive to hepcidin than WT ferroportin. (A) Effect of hepcidin on ferroportin Q248H expression. 293T cells were transfected with vectors expressing ferroportin Q248H mutant, WT ferroportin, or ferroportin C326Y (a mutant known to have reduced sensitivity to hepcidin). Ferroportin was expressed as a fusion with a c-myc–derived tag. The transfected cells were treated with the indicated concentrations of hepcidin for 1 h. Expression of ferroportin (Fpn) was analyzed by immunoblotting with anti-c-myc antibody and compared to the loading control with anti- -tubulin antibody. (B) Quantification of WT and Q248H mutant ferroportin expression. WT ferroportin and Q248H mutant ferroportin were expressed in 293T cells and analyzed as in panel A in untreated cells and cells treated with 0.03 or 0.3 μM hepcidin. Results from three independent experiments are shown. (C) Effect of ferroportin Q248H expression on cellular ferritin levels. 293T cells were transfected as in panel A and incubated with ferric ammonium citrate for 16 h. The cells were then treated with 100 μM cycloheximide for 1 h followed by treatment with 0.01 μM hepcidin for 18 h. Ferritin was measured in the cell lysates by ELISA and adjusted to total protein concentrations measured by the Bradford assay.

macrophages heterozygous for ferroportin Q248H and there was no increase in macrophages with homozygous ferroportin Q248H (Figure 3).

Characteristics of sickle cell disease patients

Of 284 patients with sickle cell anemia, 280 had hemoglobin SS and four had hemoglobin Sβ⁰-thalassemia. The mean age of these patients was 34 years (range, 18 to 74 years) and 147 (51.8%) were females. The mean (SD) hemoglobin concentration was 8.9 (1.6) g/dL and the mean (SD) reticulocyte count was 272 (127)x10⁹/L. Twenty-four (8.5%) ferroportin Q248H heterozygotes and two (0.7%) homozygotes were identified. These findings can be compared to the 8.3% rs11568350 heterozygosity rate in the Yoruban (Nigeria) (n=5/60) and the 15.8% heterozygosity rate in subjects of African ancestry (Southwest USA) (n=9/57) Hapmap populations (*www.hapmap.org*). Because of the low number of ferroportin Q248H homozyogtes, we removed them from further analyses. A subset of 78 patients with fewer than five uints of blood transfusion in their lifetimes were assessed for association of iron status with ferroportin genotype. A subset of 270 patients with measurable TRV was assessed for association of TRV with ferroportin genotype.

Measures of iron status in patients transfused with fewer than five units of blood in their lifetime

Among sickle cell anemia patients who reported receiving less than five units of blood in their lifetime, we examined indirect measures of iron status according to ferroportin genotype. Serum ferritin concentrations were lower in ferroportin Q248H heterozygotes than in those with WT ferroportin (*P*=0.018) in an unadjusted analysis (Table 2), consistent with lower macrophage iron stores in heterozygotes. The same trend persisted after adjusting for age and sex: geometric mean serum ferritin concentration of 90 μg/L in nine ferroportin Q248H heterozygotes *versus* 228 μg/L in 69 wild-type subjects (*P*=0.006). We also compared serum iron concentration and transferrin saturation according to ferroportin Q248H status in unadjusted analyses. Serum iron concentration and transferrin saturation did not differ according to ferroportin Q248H status, consistent with no decrease in plasma transport of iron by ferroportin Q248H heterozygotes despite lower serum ferritin concentration.

Association of ferroportin Q248H with tricuspid regurgitation velocity in all sickle cell anemia patients, regardless of transfusion history

In unadjusted analysis, the mean±SD TRV was 2.4±0.3 m/sec in 19 ferroportin Q248H heterozygotes *versus* 2.6±0.5 m/sec in 231 ferroportin wild-type participants (*P*=0.016). There were no ferroportin Q248H heterozygotes among 34 patients with TRV >3.0 m/sec, a cut-off that is enriched for pulmonary hypertension as defined by a mean pulmonary arterial pressure >25 mmHg deter-

Table 2. Comparison of indirect measures of iron status according to ferroportin genotype in sickle cell anemia patients who had received fewer than five blood transfusions in their lifetime.

	Q248H heterozygotes		Wildtype		P[1]
	N	Result[1]	N	Result[1]	
Ferritin (ng/ml)*	9	88 (29-273)	69	211 (74-602)	0.018
Iron (μg/dL)**	9	99 (58)	65	94 (42)	0.8
Transferrin (mg/dL)**	9	237 (53)	69	212 (47)	0.2
Transferrin saturation (%)**	9	33 (27)	65	32 (15)	0.4

[1]*Comparison by the Kruskal-Wallis test. *Geometric mean and SD range. **Mean and SD.*

Table 3. Prevalence of ferroportin Q248H according to TRV category among all sickle cell anemia patients.

	N	Ferroportin Q248H N. (%)
TRV <2.5 m/sec	116	12 (10.3%)
TRV 2.5-3.0 m/sec	100	7 (7.0%)
TRV >3.0 m/sec	34	0 (0%)
P for trend		0.050

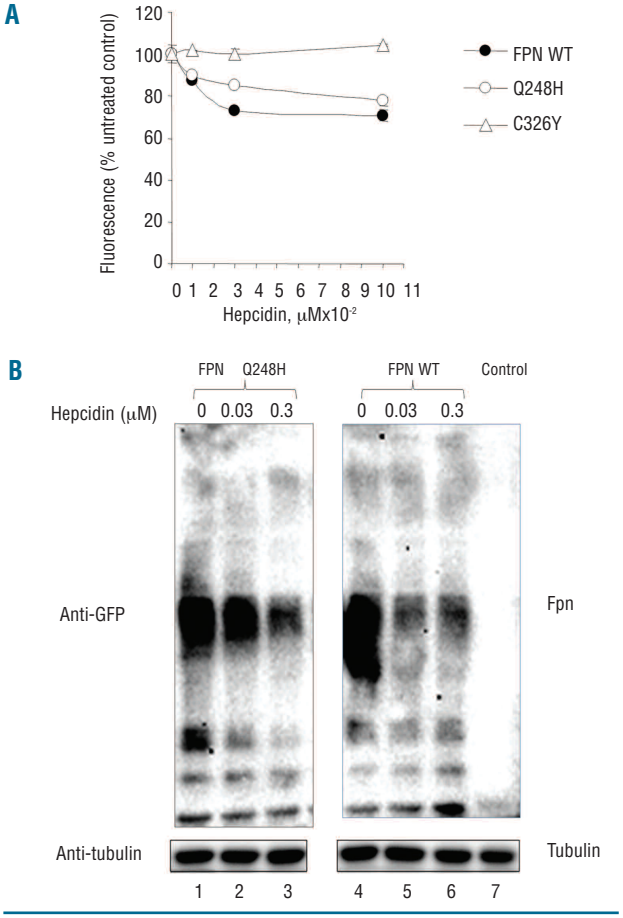

Figure 2A,B. Ferroportin Q248H-EGFP is resistant to hepcidin. Ferroportin WT and mutants Q248H or C326Y EGFP-fused proteins were expressed in 293T cells and treated with hepcidin at 37°C for 4 h. EGFP fluorescence was measured in live cells as described in the *Design and Methods* section. (A) Measurement of EGFP fluorescence showed significantly increased expression of ferroportin (FPN) C326Y-EGFP compared to FPN WT-EGFP at hepcidin concentrations of 0.01, 0.03 and 0.1 μM. FPN Q248H-EGFP was also increased at these hepcidin concentrations but less so than FPN C326Y. (B) Expression of ferroportin was analyzed by immunoblotting with anti-EGFP antibody and compared to the loading control with anti-α-tubulin antibody. Compared to ferroportin WT, ferroportin Q248H had reduced sensitivity to 0.03 μM hepcidin but not to 0.3 μM hepcidin.

mined by right heart catheterization.[36] In contrast, there were seven (7.0%) ferroportin Q248H heterozygotes among 100 patients with TRV 2.5-3.0 m/sec and 12 (10.3%) among 116 with TRV <2.5 m/sec (*P* for trend = 0.050; Table 3).

In a subanalysis, we compared TRV among ferroportin wild-type participants according to whether or not they had received five or more blood transfusions. The mean±SD TRV was 2.3±0.6 m/sec among 70 participants who had received fewer than five blood transfusions compared to 2.6±0.6 m/sec among 137 who received five or more blood transfusions (*P*=0.006).

Discussion

Our present findings suggest that the ferroportin Q248H mutant is resistant to physiological concentrations of hepcidin (0.01-0.03 μM),[12-15] despite bioinformatic predictions suggesting that it is a neutral polymorphism. We observed this with both human myc-tagged ferroportin and ferroportin fused to EGFP that were expressed in 293T cells treated with hepcidin and detected by immunoblotting or EGFP fluorescence. These observations were further supported by analysis of intracellular ferritin that reflects the amount of iron stored within the cells. The hepcidin-mediated increase of ferritin was less pronounced in 293T cells that expressed ferroportin Q248H compared to cells that expressed WT ferroportin. Analysis of ferritin levels in primary macrophages differentiated from primary human monocytes after incubation with iron and a short exposure to hepcidin showed a dose-dependent association with ferroportin genotype. No increase in macrophage ferritin was observed with homozygous ferroportin Q248H, a modest increase was observed in heterozygotes, and a maximal increase was observed in wild-type homozygotes.

Drakesmith and colleagues previously analyzed sensitivity of various murine ferroportin mutants including ferroportin Q248H to 0.5 μM hepcidin,[23] a supraphysiological concentration. While murine ferroportin Q248H was not found to be resistant to hepcidin at this concentration,[23] a systematic analysis that included lower hepcidin concentrations was not conducted. In our experiments, when compared to WT ferroportin, c-myc-fused ferroportin Q248H expressed in 293T cells was resistant to degradation by hepcidin at physiological concentrations of 0.01 μM and 0.03 μM but not at 0.1 μM, a concentration at the outer limit of the physiological range even with elevated iron stores or inflammation. In addition, we generated previously unavailable EGFP fused mutants of human ferroportin, which showed similar degradation patterns as c-myc-fused ferroportin mutants as assessed by direct measurement of fluorescence. We also extended previous studies by analyzing the effect of hepcidin in primary cells that were obtained from patients with ferroportin Q248H. In these cells we observed changes in ferritin accumulation even at a higher hepcidin concentration of 0.5 μM. It is possible that the effect of hepcidin on ferroportin expressed in cultured cells only partially recapitulates the *in vivo* situation. For example, continuous ferroportin overexpression driven by a strong cytomegalovirus promoter in the transfected cells may in principle make it less sensitive to hepcidin than when it is naturally expressed in primary cells.

Ferroportin glutamine 248 is located within the intracellular loop (residues 228-307), which presumably has a cytoplasmic localization.[37] While an early study reported that ferroportin internalization requires tyrosine phosphorylation[35] this finding was not confirmed in a more recent study which instead showed that ferroportin internalization is driven by ubiquitination of lysines lying within residues 229-269.[38] K229R/K240R/K247 mutations led to increased resistance of ferroportin to hepcidin and, importantly, the K240E mutation in humans was associated with a 2-fold increase in the hepcidin/ferritin ratio, suggesting increased iron export.[38] The proximity of the K240 and especially K247 to the Q248 residue suggests that a positively charged histidine in position 248 might change the overall negative charge of the ^{240}eeetelkqlnlhk^{253} sequence toward a more positive charge, which might affect ubiquitination and subsequent degradation of ferro-

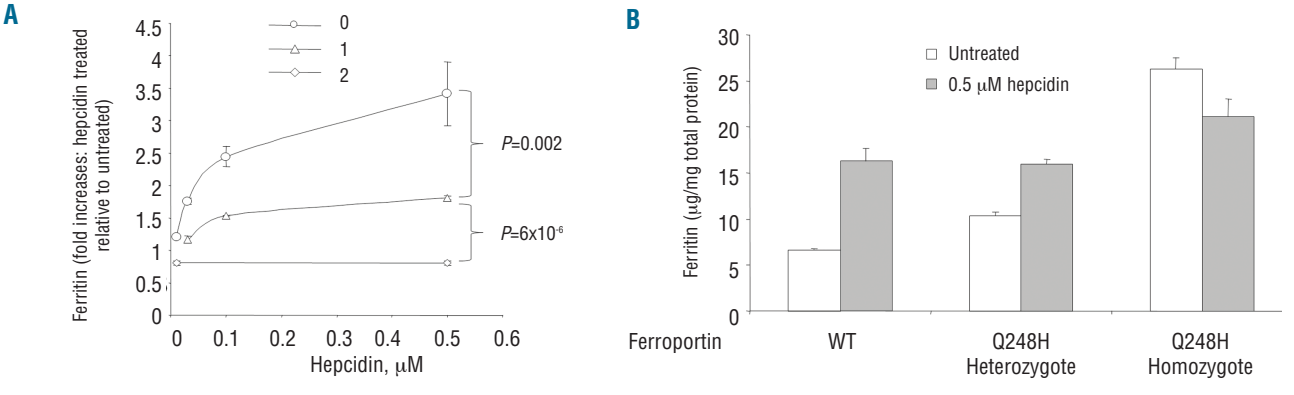

Figure 3. Lower ferritin increase in response to hepcidin in primary human macrophages expressing ferroportin Q248H. Human primary macrophages were treated with ferric ammonium citrate for 18 h and then with 0.01 -0.5 μM hepcidin concentrations for 6 h. The cells were lysed and ferritin concentrations were measured using an ELISA and adjusted to the total protein concentration determined by the Bradford assay. (A) Results are presented as fold increase in ferritin levels after hepcidin treatment relative to the levels in untreated controls. (0), macrophages expressing WT ferroportin. (1), macrophages expressing heterozygous ferroportin Q248H. (2), macrophages expressing homozygous ferroportin Q248H. (B) Ferritin concentrations are shown in untreated macrophages and macrophages treated with 0.5 μM hepcidin.

portin. Future studies should address the details of ubiquitination of human ferroportin Q248H compared to WT ferroportin.

Hepcidin is produced by hepatocytes in response to inflammatory cytokines or to increased iron stores[6,9,39,40] and then directly interacts with ferroportin on the cell membrane causing internalization of ferroportin, subsequent degradation of the ferroportin by lysosomes, and reduced export of iron from cells.[6,39] While ferroportin regulation by hepcidin applies generally to duodenal enterocytes, macrophages and other cells, export of iron by ferroportin from macrophages that phagocytose erythrocytes is additionally influenced by the catabolism of hemoglobin, which leads to up-regulation of heme oxygenase and ferroportin and to increased export of iron from macrophages.[6,7] In hemolytic anemias such as sickle cell disease, iron processing and iron export by macrophages are increased markedly by 5- to 7-fold or more compared to levels in controls.[41] We hypothesized that any effect of ferroportin Q248H in manifesting resistance to hepcidin and influencing measures of iron status might be particularly evident in patients with sickle cell anemia, especially since usually normal serum hepcidin levels and low urinary levels have been reported in this setting.[13,42,43] In contrast to population studies suggesting that ferroportin Q248H is associated with a mild increase in iron stores, as reflected by serum ferritin concentration,[17,20] we observed that ferroportin Q248H was associated with reduced serum ferritin concentration in the non- or infrequently transfused sickle cell anemia patients studied here, suggesting lower iron stores than in sickle cell anemia patients with WT ferroportin. At the same time, this allele was not associated with reduced serum iron concentrations, suggesting uninterrupted plasma transport of iron. Thus our findings are consistent with a relative resistance of ferroportin Q248H to hepcidin as indicated by our laboratory studies and paralleling the recent report of hepcidin resistance by ferroportin K240E.[38] The lower ferritin levels with ferroportin Q248H in sickle cell anemia patients are in contrast to the higher ferritin levels seen with ferroportin Q248H[17,20] and ferroportin K240E[44] in patients without hemolytic anemia. The patients with ferroportin K240E mutation also had mutations in *HFE* that may independently contribute to higher ferritin levels.[44]

A possible explanation for the lower serum ferritin concentrations in ferroportin Q248H sickle cell anemia patients in this study in contrast to the trend to higher serum ferritin concentrations in ferroportin Q248H subjects without hemolytic anemia in other studies is as follows. Ordinarily about 1 mg of dietary iron enters the circulation from enterocytes per day[45] and 20 mg of iron enters the circulation per day from macrophages that have processed phagocytosed erythrocytes.[41] However, in the setting of sickle cell anemia, this ratio may be 1 mg from the enterocytes and 100 mg or more from macrophages.[41] Resistance to hepcidin by enterocyte ferroportin Q248H would tend to favor increased iron absorption and increased total body iron stores whereas resistance to hepcidin on the part of macrophage ferroportin Q248H would tend to favor decreased macrophage iron stores, the principal site of storage iron under normal circumstances.[45] Therefore, in the context of sickle cell anemia, the effect of the resistance to hepcidin by ferroportin Q248H could be magnified in the delivery of iron from macrophages *versus* the delivery from enterocytes. Furthermore, sickle cell anemia is characterized by a certain degree of intravascular hemolysis[46] with loss of hemoglobin iron in the urine,[47] which could in part offset increased iron absorption by enterocytes. The net effect of these processes could be reduced iron stores with ferroportin Q248H in the setting of sickle cell anemia, as suggested by the results of this study.

In sickle cell anemia, the increased risk of pulmonary and systemic hypertension, renal dysfunction, proteinuria, stroke and early death may be in part related to the degree of hemolysis, nitric oxide scavenging, free radical-mediated endothelial injury and resultant damage to the vasculature.[29,30,48-51] Other risk factors associated with pulmonary hypertension and vasculopathy include left ventricular diastolic dysfunction, asplenia, cholestatic hepatic dysfunction and markers of iron overload.[29,52] Various studies have implicated pulmonary iron accumulation and/or increased body iron stores in the pathogenesis of pulmonary hypertension.[29,31,53,54] In the present study, we examined whether ferroportin Q248H may be a genetic modifier of systolic pulmonary artery pressure in sickle cell anemia. Pulmonary hypertension, as documented by right heart catheterization, is present in 6-11% of adults with sickle cell anemia.[36,55,56] Ferroportin is expressed in vascular endothelial cells,[5,57,58] and exposure of human umbilical vein endothelial cells to tumor necrosis factor-α leads to decreased ferroportin expression and an increase in iron-mediated hydroxyl radical formation.[57] Therefore, any effect that the Q248H mutation might have in enhancing ferroportin expression in pulmonary vascular endothelial cells[58] and reducing cellular iron in the setting of sickle cell anemia might be expected to provide protection from oxidant damage to the endothelium. Tricuspid regurgitation values determined by echocardiography, which reflect systolic pulmonary artery pressure,[29] were significantly lower in ferroportin Q248H heterozygotes than in subjects with both wild-type alleles. The absolute lowering in mean TRV was small (0.2 m/sec) and does not necessarily imply a clinical benefit. These findings are consistent with the possibility that ferroportin Q248H alters iron metabolism in sickle cell anemia in such a manner as to protect patients from elevated systolic pulmonary artery pressure. Additional support for an influence of iron metabolism on pulmonary artery pressure in the present study is provided by the observation that the TRV was lower in patients who had received fewer than five blood transfusions in their lifetime than in those who had received five or more blood transfusions. These observations need to be replicated in independent study populations as the results could also represent a false positive given the less than 10% frequency of ferroportin Q248H allele in our study population.

Our study has several limitations. Most of the experiments were performed *in vitro*. Perhaps, future development of a mouse model will allow *in vivo* determination of the effect of the ferroportin Q248H mutation. It will also be useful to analyze hepcidin expression levels in individuals with the ferroportin Q248H *versus* normal controls and correlate them with serum ferritin levels. In a cohort of women positive for human immunodeficiency virus, 12 individuals with the Q248H mutation had higher serum ferritin concentrations and lower serum hepcidin concentrations than 188 without the mutation; the explanation of this observation is not clear.[21] Further molecular analysis is

needed to understand the mechanism of ferroportin Q248H internalization. Measurement of ferroportin expression is challenging as it usually involves semi-reducing SD electrophoresis separation that is unlikely to resolve proteins according to their molecular weight and that puts in question the validity of the ferroportin expression measurements. Perhaps mass spectrometry analysis that has higher sensitivity and also allows direct determination of protein modification should be used to detect ferroportin expression and internalization. Recent measurement of ferroportin ubiquitination in cells with inducible ferroportin expression[59] may also be an excellent approach to analyze whether the Q248H mutation affects ferroportin ubiquitination. Taken together, our study shows that ferroportin Q248H may exert its effect due to its reduced sensitivity to hepcidin. Our study provides a mechanistic explanation for the wide prevalence of this mutation in Africa where iron deficiency and chronic infections abound.

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