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Ferroportin (*SLC40A1*) Q248H mutation is associated with lower circulating plasma tumor necrosis factor- α and macrophage migration inhibitory factor concentrations in African children

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Abstract

Background—Iron deficiency and the Q248H mutation in the gene, *SLC40A1*, that encodes for the cellular iron exporter, ferroportin, are both common in African children. The iron status of macrophages influences the pro-inflammatory response of these cells. We hypothesized that Q248H mutation may modify the inflammatory response by influencing iron levels within macrophages.

Methods—The Q248H mutation and circulating concentrations of ferritin, C-reactive protein and selected pro-inflammatory cytokines (interleukin-12, interferon- γ , TNF- α , and macrophage migration inhibitory factor) and anti-inflammatory cytokines (interleukin-4 and interleukin-10) were measured in 69 pre-school children recruited from well-child clinics in Harare, Zimbabwe.

Results—In multivariate analysis, both ferroportin Q248H and ferritin <10 ug/L were associated with significantly lower circulating concentrations of tumor necrosis factor- α . Ferroportin Q248H but not low iron stores was associated with lower circulating macrophage migration inhibitory factor as well. Anti-inflammatory cytokine levels were not significantly associated with either ferroportin Q248H or iron status.

Conclusions—Ferroportin Q248H and low iron stores are both associated with lower circulating tumor necrosis factor- α , while only ferroportin Q248H is associated with lower circulating macrophage migration inhibitory factor. Whether the reduced production of tumor necrosis factor- α observed in ferroportin Q248H heterozygotes may be of significance in anemia of chronic disease is yet to be determined.

Introduction

The *SLC40A1* gene encodes a multiple trans-membrane domain protein, ferroportin, which is responsible for iron efflux from mature enterocytes of the duodenum and from macrophages of the spleen and bone marrow to plasma [1-3]. Duodenal enterocytes are responsible for absorption of iron from the diet and macrophages are responsible for recycling iron that is recovered from the catabolism of erythrocytes that they remove from the circulation [4]. Cellular export of iron by ferroportin is regulated by hepcidin, which is

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produced by hepatocytes in response to inflammatory cytokines or to increased iron stores [5-8]. Hepcidin 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 [6,7]. The SCL40A1 mutations may be associated with predominantly parenchymal or predominantly macrophage iron-loading [9-11]. A number of disease-causing SCL40A1 gene mutations have been shown to render ferroportin resistant to hepcidin in model systems *in vitro* [12,13]. Such mutations would tend to be associated with increased iron absorption by enterocytes, increased iron-release by macrophages and parenchymal iron-loading.

The cDNA 744G>T substitution in exon 6 of the ferroportin gene (dbSNP rs11568350, www.ncbi.nlm.nih.gov), which results in the replacement of glutamine with histidine at position 248 (Q248H), is common in Africans and African Americans (prevalence of heterozygotes of 5% to 20%). Some studies have suggested association of the ferroportin Q248H with a tendency to increased iron stores in healthy adults and with possible protection from iron deficiency in children attending well child clinics [14-19].

Studies at the cellular level have shown that iron metabolism and immunity are interrelated [20]. Pro-inflammatory cytokines such as tumor necrosis factor- α (TNF- α), interleukin-1 (IL-1) or interleukin-6 (IL-6) induce increased synthesis and content of ferritin in mononuclear-phagocytic cells [21-23], whereas the anti-inflammatory cytokines such as IL-4 and IL-13 increase macrophage expression of transferrin receptors [24]. At the systemic level, studies in experimental animals and humans have shown that administration of TNF- α suppresses serum iron concentration [25-27]. Not only does inflammation affect iron metabolism, but iron status itself may modulate the production of inflammatory cytokines [28-30]. Iron deficiency anemia in infants was reported to increase lipopolysaccharide-induced production of tumor necrosis factor- α by peripheral blood mononuclear cells [29]. In contrast to this report, peripheral blood mononuclear cells from patients with hereditary hemochromatosis, a condition with paradoxically low iron concentration in such cells, released less TNF- α than peripheral blood mononuclear cells from controls [31], and iron supplementation of peripheral blood mononuclear cells from patients with iron deficiency anemia led to increased mRNA expression of TNF- α , IL-6 and IL-10 [32]. Several studies have found a relationship between reduced iron status and decreased NF- κ B-mediated TNF- α production by rodent hepatic macrophages [33,34]. A peroxynitrite-mediated transient rise in intracellular labile iron may be a signal for endotoxin-induced I κ B kinase (IKK) and NF- κ B activation in rodent [35] and human macrophages [36] leading to TNF- α expression; this process is blocked by iron chelation.

Why the ferroportin Q248H allele has achieved a high frequency in African populations is not known, but one possibility is that it in some manner influences immune responses in a favorable manner through altering iron levels in macrophages and/or serum. In this study we investigated the effect of the ferroportin Q248H mutation on plasma cytokine concentrations.

Subjects and Methods

Study participants

Subjects were selected from a study of iron deficiency in African children that has been described previously [16]. Briefly, 208 apparently healthy children aged ≤ 60 months attending well-child clinics in Harare, Zimbabwe were enrolled. Five milliliters of peripheral blood were drawn from each child during the morning into two vacutainer tubes, one containing K₃-EDTA and one with no anticoagulant. In the current study, only 69 children (37 females) had sufficient plasma to perform cytokine measurements. Ethical permission

was granted by the Medical Research Council of Zimbabwe and the Howard University IRB and written permission was obtained from the mothers or guardians.

Laboratory measurements

Complete blood counts were performed on an automated analyzer (Sysmex, Norderstedt, Germany). Serum ferritin and C-reactive protein (CRP) concentrations were measured using enzyme immunoassays with commercially available kits (Ramco Laboratories, Stafford, TX and ALPCO Diagnostics, Windham, NH, respectively). Plasma samples were assayed in duplicate and concentrations of IL-4, IL-10, IL-12, interferon- γ and TNF- α were measured using Human Cytokine/Chemokine Multiplex Immunoassay kits (LINCO Research, St. Charles, MO, USA). Plasma concentrations of macrophage migration inhibitory factor (MIF) were measured using Human Sepsis/Apoptosis Multiplex Immunoassay kits (LINCO Research, St. Charles, MO, USA).

The *SLC40A1* Q248H mutation was identified as previously reported [16]. In brief, DNA was isolated from leukocytes obtained from whole blood using lymphocyte separation media (Media-Tech, Stirling, VA). Exon 6 of ferroportin gene was amplified using a set of primers (forward primer: 5'-CAT CGC CTG TGG CTT TAT TT-3'; reverse primer 5'-GCT CAC ATC AAG GAA GAG GG-3') in a PTC- 100 thermocycler (MJ Research Inc. Waltham, MA) to make a 392bp product. The 392bp product was digested by *PvuII* enzyme (MBI Fermentas, Hanover, MD) and the resulting DNA fragments (252bp, 140bp) were separated on 3% agarose gel and detected with ethidium bromide.

Definition of iron status

Low iron stores was defined as serum ferritin concentration of $<10 \mu\text{g/L}$ [37]. C-reactive protein concentrations $>8.2 \text{ mg/L}$ were taken to be elevated as provided by the manufacturer of the kit. Population reference values for C-reactive protein are not available for this population.

Statistical analysis

Statistical analysis was performed with SYSTAT software (version 11; SYSTAT Software, Inc, Point Richmond, CA). Clinical variables were compared according to ferroportin Q248H mutation status with the Kruskal-Wallis test for continuous variables and the Fisher exact test for proportions. Because age varied significantly according to iron status, continuous variables were compared according to iron status by analysis of variance with adjustment for age and categorical variables were compared by logistic regression with adjustment for age. To determine the independent associations of circulating cytokine concentrations with ferroportin Q248H and iron status, we constructed multivariate linear regression models. For parametric analyses, variables that followed a skewed distribution were log transformed.

Results

Circulating cytokine concentrations according to ferroportin Q248H

Table 1 summarises the laboratory data of the study population. The median serum ferritin median concentration was $19 \mu\text{g/L}$ (interquartile range: $11\text{--}33 \mu\text{g/L}$). The median TNF- α concentration was 16.02 pg/mL (interquartile range: $5.14\text{--}20.14 \text{ pg/mL}$) whereas the median MIF concentration was 13.68 ng/mL (interquartile range: $4.56\text{--}26.30 \text{ ng/mL}$). Fourteen (22%) of 64 children were heterozygous for ferroportin Q248H. In five children ferroportin genotype was not determined due to poor quality of the DNA recovered. Clinical features and plasma cytokine concentrations are presented in Table 2 according to ferroportin Q248H status. Concentrations of tumor necrosis factor- α and macrophage migration inhibitory

factor were significantly lower in the patients with ferroportin Q248H. Serum ferritin was $<10 \mu\text{g/L}$ in 7% of the children with ferroportin Q248H versus 24% of those with ferroportin wildtype, but this difference did not reach statistical significance.

Circulating cytokine concentrations according to iron status

Thirteen (19%) of 69 children had serum ferritin concentration $<10 \mu\text{g/L}$. Clinical features and plasma cytokine concentrations are presented in Table 3 according to serum ferritin concentration $<10 \mu\text{g/L}$ versus $\geq 10 \mu\text{g/L}$. Children with serum ferritin $<10 \mu\text{g/L}$ were significantly younger than the children with higher serum ferritin concentration. Because of this, the clinical features and cytokine levels were compared according to iron status category in analyses that adjusted for age. The age-adjusted plasma TNF- α concentration were significantly lower in the children with lower ferritin concentration, but the macrophage migration inhibitory factor concentrations did not differ significantly according to iron status. The Q248H allele was present in 8% of the children with ferritin $<10 \mu\text{g/L}$ compared to 25% of those with ferritin concentrations of $10 \mu\text{g/L}$ or higher.

Independent relationship of circulating cytokine concentrations to ferroportin Q248H and iron status by multivariate analysis

We examined the relationships of both serum ferritin concentration $<10 \mu\text{g/L}$ and ferroportin Q248H with log TNF- α in a single linear regression model that adjusted for age and log C-reactive protein concentration. The independent associations of both low iron status ($P = 0.015$) and the ferroportin Q248H allele ($P = 0.007$) with lower plasma TNF- α concentration were confirmed in this model as shown in Table 4. In a similar model, ferroportin Q248H but not low iron status was associated with lower plasma MIF concentration (geometric mean MIF concentration of 5.7 ng/mL with ferroportin Q248H versus 14.9 ng/mL with ferroportin wildtype; $P = 0.013$).

Discussion

In this study with a limited sample size, we observed lower plasma concentrations of TNF- α in African pre-school children with the ferroportin Q248H mutation or with low iron status. We also observed lower concentrations of MIF in association with ferroportin Q248H but not with low iron status. Tumor necrosis factor- α is an important mediator of the inflammatory process and is produced predominantly by activated monocytes/macrophages [38,39]. Several mechanisms control the secretion of TNF- α . NF- κB plays a role in expression of proinflammatory genes including TNF- α gene [reviewed in 40], but lipopolysaccharide (LPS) and viruses have been shown to induce the TNF- α gene independently of NF- κB [41]. Macrophage migration inhibitory factor is a pro-inflammatory cytokine that is produced by activated macrophages and plays a role in the systemic inflammatory response by counter-regulating the inhibitory effects of glucocorticoids on TNF- α and IL-6 production [42]. The effect of ferroportin Q248H mutation on plasma concentrations of TNF- α and MIF in this study could not seem to be attributed to inflammation, for it persisted after adjustment for the C-reactive protein concentration.

Since monocytes and macrophages are major sources of circulating TNF- α , the findings we report here are consistent with low iron stores in children limiting TNF- α production by macrophages. These results are also consistent with the possibility that ferroportin Q248H in children is associated with increased export of iron from macrophages, reduced intracellular labile iron concentration, and consequent decreased production of TNF- α . One *in vitro* study indicated that the Q248H allele impairs the egress of iron when expressed in *Xenopus* oocytes [43]. Other studies indicated that the Q248H allele retains the ability to export iron and respond to hepcidin when expressed in HEK 293T cells [12,13]. In fact, Drakesmith and

colleagues found that ferroportin Q248H was as susceptible to 0.5 μ M hepcidin as wildtype ferroportin in this experimental setting, but commented that this polymorphism may have a mild effect on ferroportin function that they could not detect and possibly lead “to disease in the presence of modifying factors” [12,13].

Thus, as postulated by Schimanski et al, Q248H mutation in SCL40A1 gene may result in gain of function by ferroportin leading to depletion of macrophage cellular iron [13]. This would lead to reduced macrophage cellular iron which in turn would lead to reduced activation of the transcription factor NF- κ B and consequently reduced TNF- α production [33]. As a result, macrophage TNF- α production is reduced in persons with ferroportin Q248H. Conversely, increased iron content in Kupffer cells promotes activation of NF- κ B and subsequent induction of TNF- α expression [35,44]. We have recently demonstrated that TNF- α release is enhanced in iron-laden macrophages derived from human blood monocytes due to accentuated intracellular labile iron [36]. However, to our knowledge no studies have demonstrated that macrophages obtained from ferroportin Q248H heterozygotes have lower intracellular iron content compared to macrophages obtained from ferroportin wildtype persons.

Low iron stores, plasma ferritin < 1 μ g/L, was associated with lower plasma TNF- α concentration. Similar findings were reported by Wang et al [45]. In experimental studies on Hfe knockout mice reduced intra-macrophage iron resulted in decreased TNF- α secretion [45]. In this study, ferroportin Q248H was associated with lower concentration of MIF compared to ferroportin wildtype. Secretion of MIF is mediated by several pathways. In one pathway, TNF- α induces MIF gene expression resulting in elevated levels of circulating plasma MIF [46]. This mechanism could explain the observed correlation of TNF- α plasma levels with MIF concentration. Thus, ferroportin Q248H which is associated with lower TNF- α concentration would have correspondingly lower MIF levels.

The biological significance of the ferroportin Q248H mutation which is unique and prevalent in African populations is yet to be determined. Iron deficiency is endemic in sub-Saharan Africa due to poor dietary iron sources and chronic hookworm infestations [47]. In the parent study, we proposed that the ferroportin Q248H mutation may be protective against iron deficiency in children exposed to repeated infections [16]. In recent studies in mice, TNF- α was implicated in the pathogenesis of anemia of chronic disease by enhancing iron sequestration in spleen macrophages, by reducing iron transfer from duodenal enterocytes [48] and by inducing hypoferraemia [27]. Furthermore, increased circulating TNF- α concentration induces macrophage iron accumulation through increased expression of divalent metal transporter-1 and down-regulation of ferroportin expression [23] probably due to TNF- α -enhanced hepatocellular production of hepcidin which binds to ferroportin causing its internalization and degradation leading to decreased release of recycled iron by macrophages. Conversely, reduced circulating TNF- α concentration would lead to reduced hepcidin production and hence decreased internalization of ferroportin [49]. However, in this study hepcidin concentration was not measured.

If findings of our study are corroborated in a study with a larger sample size, we hypothesize that ferroportin Q248H mutation may contribute to lower plasma TNF- α concentration. As a result iron recycling by macrophages would not be severely impaired in chronic inflammation. However, we were unable to measure serum iron concentration in this population due to insufficient sample volume. Whether the reduced production of TNF- α and subsequently decreased MIF concentration associated with ferroportin Q248H mutation may be of significance in anemia of chronic disease is yet to be determined.

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Table1
Summary data of the study population

	n	Median (IQR)
Age, months	69	24 (16 - 39)
White blood cell, $\times 10^3/\mu\text{L}$	62	7.9 (6.1 - 10.9)
Hemoglobin, g/dL	62	10.9 (10.3 - 11.6)
C-reactive protein, mg/L	69	0.66 (0.14 - 2.01)
Serum ferritin, $\mu\text{g/L}$	69	19 (11 - 33)
Interleukin-4, pg/ml	68	2.0 (2.0 - 132.4)
Interleukin-10, pg/ml	68	1.1 (1.1 - 26.6)
Tumor necrosis factor- α , pg/mL	68	16.0 (5.1 – 20.1)
Macrophage migration inhibitory factor, ng/mL	68	13.7 (4.6 – 26.3)
Interferon- γ , pg/ml	68	0.7 (0.7-0.7)
Interleukin-12, pg/ml	68	0.5 (0.5-0.5)

Table 2
Comparison of clinical features and cytokine profile according to ferroportin Q248H status

	Ferroportin wild type		Ferroportin Q248H heterozygotes		P*
	N	median (IQR)	n	Median (IQR)	
Age, months	50	23 (16-39)	14	29 (16-36)	0.661
White blood cell, $\times 10^3/\mu\text{L}$	46	8.3 (6.2-10.9)	13	7.6 (6.4-10.4)	0.812
Hemoglobin, g/dL	46	10.9 (10.3-11.5)	13	11.2 (10.6-12.0)	0.272
C-reactive protein, mg/L	50	0.54 (0.10-1.97)	14	0.96 (0.28-2.04)	0.217
Serum ferritin, $\mu\text{g/mL}$	50	20 (10-33)	14	17 (11-29)	0.948
Serum ferritin $<10 \mu\text{g/L}$, n (%)	50	12 (24.0%)	14	1 (7.1%)	0.266
Interleukin-4, pg/mL	50	2.0 (2.0-135.6)	14	2.0 (2.0-94.7)	0.889
Interleukin-10, pg/mL	50	1.1 (1.1-25.9)	14	1.1 (1.1-45.7)	0.567
Tumor necrosis factor- α , pg/mL	49	17.0 (11.4-19.8)	14	7.5 (0.3-15.1)	0.018
Macrophage migration inhibitory factor, ng/mL	49	20.0 (6.0-37.0)	14	5.7 (2.8-10.7)	0.002
Interferon- γ , pg/mL	50	0.7 (0.7-0.7)	14	0.7 (0.7-0.7)	0.845
Interleukin-12, pg/mL	50	0.5 (0.5-0.5)	14	0.5 (0.5-0.5)	0.346

* Comparison by the non-parametric Kruskal-Wallis test for continuous variables and the Fisher exact test for proportions.

Table 3
Comparison of clinical features and cytokine profile according to serum ferritin <10 µg/L versus ≥10 µg/L

	N	Ferritin ≥10 µg/L	n	Ferritin <10 µg/L	p*
Age, months	56	30 (18-42)	13	18 (11-20)	0.002
White blood cells, ×10 ³ /µL	50	7.6 (5.9-10.3)	12	9.8 (7.5-11.0)	0.571
Hemoglobin, g/dL	50	11.2 (10.5-11.8)	12	10.4 (9.1-10.9)	0.077
C-reactive protein, mg/L	56	0.85 (0.16-2.34)	13	0.29 (0.7-0.61)	0.930
Ferroportin Q248H, n (%)**	51	13 (25.5%)	13	1 (7.7%)	0.191
Interleukin-4, pg/ml	55	2.01 (2.01-122.53)	13	2.01 (2.01-357.83)	0.713
Interleukin-10, pg/ml	55	1.09 (1.09-30.7)	13	1.09 (1.09-23.22)	0.134
Macrophage migration inhibitory factor, ng/ml	55	13.2 (3.6-26.2)	13	20.0 (5.7-32.9)	0.605
Tumor necrosis factor- α, pg/ml	55	16.0 (11.4-20.2)	13	16.5 (0.3-19.5)	0.019
Interferon-γ, pg/ml	55	0.67 (0.67-0.67)	13	0.67 (0.67-0.67)	0.262
Interleukin-12, pg/ml	55	0.48 (0.48-0.48)	13	0.48 (0.48-0.48)	0.625

* Comparison by ANOVA unless otherwise indicated. All analyses except age are adjusted for age.

** Comparison by logistic regression.

Results are expressed as median (interquartile range) unless otherwise specified.

Table 4
Independent negative associations of ferroportin Q248H and low iron stores with TNF- α in a linear regression model adjusted for age and C-reactive protein

	N	TNF- α (pg/ml) (least squares geometric mean and SD range)	P
Ferroportin			0.007
Q248H	14	1.7 (1.0-2.7)	
Wildtype	49	6.6 (5.1-8.5)	
Iron status			0.015
Ferritin <10 ug/L	14	1.7 (1.1-2.8)	
Ferritin $\geq$ 10 ug/L	49	6.4 (4.9-8.3)	