Published in final edited form as:
Blood Cells Mol Dis. 2007 ; 38(3): 247–252. doi:10.1016/j.bcmd.2006.12.002.

Association of ferroportin Q248H polymorphism with elevated levels of serum ferritin in African-Americans in the Hemochromatosis and Iron Overload Screening (HEIRS) Study

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Abstract

The ferroportin (*FPN1*) Q248H polymorphism has been associated with increased serum ferritin (SF) levels in sub-Saharan Africans and in African Americans (AA). AA participants of the HEIRS Study who did not have *HFE* C282Y or H63D who had elevated initial screening SF (≥300 μg/L in men and ≥200 μg/L in women) (defined as cases) were frequency-matched to AA participants with normal SF (defined as controls) to investigate the association of the Q248H with elevated SF. 10.4% of cases and 6.7% of controls were Q248H heterozygotes (P = 0.257). Q248H homozygosity was observed in 0.5% of the cases and none of the controls. The frequency of Q248H was higher among men with elevated SF than among control men (P = 0.047); corresponding differences were not observed among women. This appeared to be unrelated to self-

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reports of a previous diagnosis of liver disease. Men with elevated SF were three times more likely than women with elevated SF to have Q248H (P = 0.012). There were no significant differences in Q248H frequencies in men and women control participants. We conclude that the frequency of the *FPN1* Q248H polymorphism is greater in AA men with elevated SF than in those with normal SF.

Keywords

genetics; mutation; transferrin saturation

Introduction

The ferroportin (*FPN1*) Q248H polymorphism (cDNA 744 G→T [Gln248His, Q248H]) occurs in sub-Saharan African Natives and in African Americans (AA) with and without iron overload [1-3]. However, the possible role of Q248H in the causation of increased serum ferritin concentration (SF) or iron overload in sub-Saharan African Natives or AA is incompletely defined. Among 22 southern African first-degree family members, 10 of whom were Q248H heterozygotes, Q248H was associated with a trend of higher SF to aspartate aminotransferase ratios [3]. In 278 AA males and 293 AA females from a southern California screening program who lacked *HFE* C282Y, the mean SF of 26 Q248H heterozygotes did not differ significantly from that of wild-type homozygotes [2]. When the distribution of genotypes among these subjects was stratified to SF of more or less than 500 μg/L in men, and more or less than 350 μg/L in women, a X^2 test for trend was highly significant [2].

The HEIRS Study is an on-going investigation to evaluate the prevalence, genetic and environmental determinants, and potential clinical, personal and societal impact of iron overload and hemochromatosis in a multi-center, multi-ethnic, primary care-based sample of 100,000 adults ≥25 years of age [4]. All participants were screened by performing serum biochemical tests of iron status (serum transferrin saturation, SF) and genotyping for the common C282Y and H63D mutations of the hemochromatosis gene (*HFE*). We hypothesized that the frequency of the *FPN1* Q248H polymorphism in AA HEIRS Study participants with elevated SF is higher than that in AA participants with normal SF. Therefore, we selected AA participants who did not have *HFE* C282Y or H63D, and compared the prevalence of Q248H in participants with elevated SF with that in participants whose SF was normal. The possible association of Q248H with elevated SF and with iron overload is discussed.

Methods

Recruitment of participants

The design of the HEIRS Study has been reported in detail [4]. All AA HEIRS Study participants were recruited from public and private primary care settings at these HEIRS Field Centers: Howard University (Washington, D.C.), University of Alabama at Birmingham (Birmingham, AL), University of California, Irvine (Irvine, CA), and Kaiser Permanente (Portland, OR). All participants were ≥25 years old and gave informed consent.

HFE genotype analyses and serum ferritin measurements

HFE C282Y and H63D were assessed in DNA from the buffy coat of whole-blood EDTA samples by a modification of the Invader® assay (Third Wave Technologies, Inc., Madison, WI) [5,6]. For the present analyses, we included only subjects who had neither the C282Y nor H63D mutations of *HFE*.

The methods and quality assurance procedures used for measurements of SF in the HEIRS Study are described in detail elsewhere [6]. The HEIRS Study defined these initial screening SF phenotypes to be elevated: ≥300 μg/L for men and ≥200 μg/L for women [4].

Participant selection criteria for FPN1 Q248H polymorphism detection

Participants were first selected by multiple linear regression using all data from all participants in the initial screening phase whose HEIRS Study data sets were complete; the natural logarithm of SF was the response variable, and race/ethnicity, Field Center, *HFE* genotype, gender, and age were the explanatory variables. Resultant residuals were calculated for each participant from this regression. AA men and women with the *HFE* genotype wt/wt who had the highest residuals were selected; most of these participants (98.9%) were recruited from Field Centers at Howard University and the University of Alabama at Birmingham. Of the 27,124 AA HEIRS Study initial screening participants, 222 were selected as cases for the present study because they met the aforementioned elevated SF and *HFE* wt/wt criteria.

Control AA HEIRS Study participants were randomly selected and frequency-matched to cases with respect to Field Center and to clinic group within respective Field Centers. The control participants were refined further by selecting only those participants (N = 210) who had normal SF, *i.e.*, >25 to <300 μg/L in men and >15 to <200 μg/L in women; all control subjects had *HFE* wt/wt.

FPN1 Q248H polymorphism detection with PCR-RFLP

Exon 6 of *FPN1* was amplified using the touch-down method of PCR amplification: the annealing temperature was dropped from 60°C to 50°C over a period of 20 cycles, followed by 10 more cycles at 50°C, a modification of amplification conditions previously reported [7]. Amplification was performed in a 25 μL volume using 50 ng of soluble template DNA or a 1.5-mm punch of FTA®-treated paper (Whatman, Clifton, NJ) containing participant DNA. The amplification cocktail consisted of 1X Thermopol II buffer (New England Biolabs, Beverly, MA), 3.2 mM $MgSO_4$, 1 μM of each primer, 200 μM dNTP's, and 0.5 U Platinum Taq Polymerase (Invitrogen, Carlsbad, CA).

Amplified material was subsequently digested overnight with the restriction endonuclease Pvu II (New England Biolabs), which digests amplicons carrying the wild-type sequence into two easily resolvable bands on 2% agarose. However, Pvu II does not digest amplified material carrying the mutation leading to the Q248H polymorphism, thus leaving a single band of the original amplicon size. A previously sequenced and independently confirmed heterozygous control specimen [2] was included in all amplifications and digests. All heterozygous and homozygous samples for the *FPN1* Q248H polymorphism were re-tested by PCR-RFLP. Results failing to match on repeat testing were sequenced.

FPN1 Q248H polymorphism detection using sequence-based typing

The *FPN1* genotype determined by PCR-RFLP technique was inconclusive in three cases that were further evaluated by sequencing. New primers were developed to amplify a 147 base-pair region enclosing the single nucleotide transversion mutation site responsible for the Q248H polymorphism. Amplification was performed in a 10 μL volume using 50 ng of soluble template DNA or a 1.5 mm punch of FTA®-treated paper containing participant DNA. The amplification cocktail consisted of 1X Thermopol I buffer, which contained 2 mM $MgSO_4$ at 1X, 1 μM each of forward primer 5'-cgttctgctctggaaggttt-3' and reverse primer 5'-ggtctgaacatgagaacaaaagg-3', 200 μM of each dNTP, and 0.5 U Taq DNA Polymerase (New England Biolabs, Beverly, MA). Cycle parameters were a single cycle of 94°C for 2 minutes, followed by 25 cycles of 94°C for 15 seconds, 64°C for 15 seconds,

72°C for 45 seconds, and a final extension of 72°C for 5 minutes. PCR products were prepared and sequenced using the Sequenase v2.0 PCR Sequencing Kit (USB, Cleveland, OH) utilizing ^{32}P-α-dATP radioisotope incorporation according to the manufacturer's directions.

Sequenced samples were heated to >99°C for 2.5 minutes in formamide-based loading dye, then snap-cooled on ice before loading onto a 8% acrylamide (19:1 acrylamide:bis) / 32% formamide / 5.6M urea denaturing gel. Bands were separated by electrophoresis at 80W-100W for 4 hours in 1X TBE. After electrophoresis, gels were exposed to autoradiography film without intensifying screens for 16 hours at −80°C and then developed. Participants without *FPN1* Q248H were designated to have a *FPN1* wild-type genotype (wt/wt).

Self-reported conditions

The HEIRS Study collected self-reported information on arthritis, diabetes mellitus, and liver disease as part of an Initial Screening questionnaire [4]. Because the self-report information was not included in the aforementioned case and control selection, these self-reported conditions were analyzed between participants with elevated SF and controls, among and between genders, using Fisher's exact test.

Statistical analyses

FPN1 Q248H genotype frequencies in cases and controls were compared using Fisher's exact test and logistic regression. The evaluation of the association of elevated SF with Q248 was accomplished by logistic regression modelling. The models were fitted with case or control status as the binary outcome variable and the following as covariates: age, gender, presence or absence of Q248H, and presence or absence of the *FPN1* Q248H polymorphism. SF values were converted to natural logarithms to generate the summary values, because SF values are not normally distributed; the resulting antilogs were computed for presentation of final SF values. Hardy-Weinberg distributions of ferroportin genotypes were not assumed in the present study, because the cases were not randomly selected from the general HEIRS Study population sample. Descriptive data are displayed as enumerations, percentages, allele frequencies or mean ± 1 SD. P-values (alpha at 5%), odds ratios (OR) and 95% confidence intervals (95% CI) are reported (SAS v9.1, SAS Institute Inc., Cary, NC). All reported P-values are two-tailed.

Results

Characteristics of participants

The proportions of women in case and control groups were greater than the proportions of men in these respective groups (Table 1); this is consistent with the greater proportion of women than men who participated in the HEIRS Study [5]. The mean ages of men and women in case and control subgroups did not differ significantly (P = 0.859). The percentages of women in case and control subgroups who reported having arthritis were significantly greater than the percentages of men in the respective subgroups who reported having arthritis (P = 0.016). Approximately one-fifth of the present subjects reported having diabetes mellitus. In cases and controls, the proportion of men who reported having diabetes was not significantly different from that of women (cases P = 0.075; controls P = 0.286). Liver disease was reported by less than 1% of control participants, whereas 12.8% of cases reported having liver disease (P <0.001) (Table 1).

Relationship of serum ferritin and FPN1 genotype

Summary, quartile ranges of SF values, and *FPN1* genotype counts of case and control participants, stratified by gender were evaluated. In men, mean SF values were 812 μg/L (range 304 - 6,450 μg/L) in cases and 131 μg/L (range 36 - 286 μg/L) in controls. In women, mean SF values were 623 μg/L (range 201 - 4,517 μg/L) in cases and 53 μg/L (range 7.5 - 193 μg/L) in controls. Genotype frequencies did not differ significantly across quartiles in any of the case/control and gender subgroups. Therefore, Q248H distributions were analyzed solely on the basis of case or control status. The genotype distributions of *FPN1* Q248H between cases and controls, grouped by gender, are presented in Table 2. Overall, 23 of 222 (10.4%) cases with elevated SF were Q248H heterozygotes compared to 14 of 210 (6.7%) of controls. One (0.5%) of the cases and none of the controls had Q248H homozygosity (P = 0.257).

SF selection criteria for cases and controls in the present study differed in men and women, so we evaluated Q248H frequencies in men and women separately. In men, cases were three times more likely to have Q248H (18/106; 17.0%) than controls (3/60; 5%); this difference was significant after adjusting for age (P = 0.047). In contrast, the prevalence of Q248H in female cases (6/116; 5.2%) did not differ significantly from that in female controls (11/150; 7.3%) (P = 0.645). The mean age of the women in the control group (48 ± 15 y) was lower than that of the women in the case group (54 ± 14 y, P <0.001), whereas there was no significant difference in the mean age of men in the two groups (51 ± 11 y and 50 ± 14 y, respectively; P = 0.505).

Relationship of self-reports of liver disease, FPN1 Q248H, and serum ferritin levels

Of the 432 participants (166 men, 266 women), 89.4% (143 men, 243 women) answered the initial screening question regarding a previous diagnosis of liver disease (Table 3). Respective mean SF values were higher in men and women who reported that they had been previously diagnosed to have liver disease than in men and women who reported not having liver disease (Table 3). None of the 26 participants who reported that they had liver disease had Q248H; this proportion did not differ significantly from the proportion of the participants who reported that they did not have a previous diagnosis of liver disease who had Q248H (Table 3). Among subjects who reported that they did not have a previous diagnosis of liver disease, the prevalence of men with Q248H was significantly higher than the prevalence of women with Q248H (14.4% vs. 7.5%, respectively; P = 0.0345, X^2 test).

Discussion

In the present study, the frequency of *FPN1* Q248H was greater in AA men with elevated SF than in those with normal SF. This is consistent with our hypothesis that SF is positively associated with Q248H, and with previous reports [2,3]. Further, the occurrence of Q248H in AA could partly explain the higher mean SF levels typically observed in AA than in whites [1-3]. However, a corresponding difference in Q248H frequency between women with elevated SF and those with normal SF was not observed.

It is plausible that ferroportin interacts with a gender-specific trait. There are gender-related differences in the iron phenotypes of normal persons and in those with heritable iron overload disorders [8-12], and allele/haplotype frequency disparities between men and women with hemochromatosis and other disorders [13,14]. Ferroportin interacts with hephaestin, an X-linked iron transporter, to transport iron out of enterocytes [15,16]. Female mice produce more hepcidin mRNA and have higher iron levels in liver and spleen than male mice [17]; this could also account for sex-associated differences in the function of normal or abnormal ferroportin as an iron exporter. Altogether, the molecular basis by which

ferroportin Q248H affects iron metabolism is unclear, expression of Q248H in *Xenopus* oocytes decreased the efficiency of iron export, but did not alter ferroportin regulation by hepcidin [18].

The percentage of the present participants with hyperferritinemia who reported having liver disease was significantly higher than that of control subjects. In addition, the prevalence of liver disease self-reports was significantly greater in men than women, and the frequency of Q248H was greater in men with elevated SF than in those with normal SF. There are two possible explanations for these observations. The first is that Q248H modifies the susceptibility to or expression of common liver disorders such as excess ethanol ingestion, steatosis, or viral hepatitis or other conditions that are associated with hyperferritinemia. In sub-Saharan African Native children, for example, there was a significant complementary interaction of Q248H and C-reactive protein to increase SF [19]. However, the proportion of the present subjects who had Q248H did not differ significantly in those who reported and in those who did not report a previous diagnosis of liver disease. The second possibility is that Q248H has no causal relationship to common forms of hepatic disorders, and thus the increased prevalence of Q248H among AA men with hyperferritinemia is independent of the increased percentage of men designated as cases who reported that they had liver disease. This possibility is supported by our observations that the prevalence of men with Q248H was significantly higher than the prevalence of women with Q248H among the participants who reported that they did not have a previous diagnosis of liver disease. However, obtaining self-reports of alcohol consumption or performing evaluations for liver disorders or other inflammatory conditions that could explain hyperferritinemia was not included in initial screening in the HEIRS Study [4].

Iron overload is relatively common in AA [20-24], but is rarely attributable to homozygosity for *HFE* C282Y [6,24]. In the present study, all participants lacked C282Y, but some participants classified as cases also had markedly elevated levels of transferrin saturation. Therefore, it is possible that some of these participants had primary iron overload, although measurement of body iron stores by quantitative phlebotomy or analyses of hepatic biopsy specimens was beyond the scope of the present investigation. In a study of 19 families with African iron overload, there was no evidence that *FPN1* Q248H was responsible for the condition [25], and the frequency of Q248H in Alabama AA with and without primary iron overload was similar [1]. Altogether, there is little evidence that Q248H causes iron overload, although Q248H may protect against iron deficiency [25].

Acknowledgments

The HEIRS Study was initiated and funded by the National Heart, Lung, and Blood Institute, in conjunction with the National Human Genome Research Institute. The Study is supported by contracts N01-HC05185 (University of Minnesota); N01-HC05186, N01-CM-07003-74, and Minority CCOP (Howard University); N01-HC05188 (University of Alabama at Birmingham); N01-C05189 (Kaiser Permanente Center for Health Research); N01-HC05190 (University of California, Irvine); N01-HC05191 (London Health Sciences Centre); and N01-C05192 (Wake Forest University). Additional support was provided by the University of Alabama at Birmingham General Clinical Research Center (GCRC) grant M01-RR00032, Southern Iron Disorders Center (J.C.B.), Howard University GCRC grant M01-RR10284, Howard University Research Scientist Award UH1-HL03679-05 from the National Heart, Lung, and Blood Institute and the Office of Research on Minority Health (V.R.G.); and the General Clinical Research Center, College of Medicine, University of California, Irvine, with funds provided by the National Center for Research Resources, 5M01RR 00827-29, U.S. Public Health Service.

We acknowledge the following participating HEIRS Study Investigators and Institutions:

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Birmingham, AL--University of Alabama at Birmingham:

Dr. Ronald T. Acton (Principal Investigator), Dr. James C. Barton (Co-Principal Investigator), Ms. Deborah Dixon, Dr. Susan Ferguson, Dr. Richard Jones, Dr. Jerry McKnight, Dr. Charles A. Rivers, Dr. Diane Tucker and Ms. Janice C. Ware.

Irvine, CA--University of California, Irvine:

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London, Ontario, Canada--London Health Sciences Center:

Dr. Paul C. Adams (Principal Investigator), Ms. Erin Bloch, Dr. Subrata Chakrabarti, Ms. Arlene Fleischhauer, Ms. Helen Harrison, Ms. Bonnie Hogan, Ms. Kelly Jia, Dr. John Jordan, Ms. Sheila Larson, Dr. Edward Lin, Ms. Melissa Lopez, MDS Laboratories, Dr. Godfrey Moses, Ms. Lien Nguyen, Ms. Corry Pepper, Dr. Tara Power, Dr. Mark Speechley, Dr. Donald Sun and Ms. Diane Woelfle.

Portland, OR and Honolulu, HI--Kaiser Permanente Center for Health Research, Northwest and Hawaii, and Oregon Health and Science University:

Dr. Emily L. Harris (Principal Investigator), Dr. Mikel Aickin, Dr. Elaine Baker, Ms. Marjorie Erwin, Ms. Joan Holup, Ms. Carol Lloyd, Dr. Nancy Press, Dr. Richard D. Press, Dr. Jacob Reiss, Dr. Cheryl Ritenbaugh, Ms. Aileen Uchida, Dr. Thomas Vogt and Dr. Dwight Yim.

Washington, D.C.--Howard University:

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NHLBI Project Office

Bethesda, MD--Ms. Phyliss Sholinsky (Project Officer), Dr. Ebony Bookman, Dr. Henry Chang, Dr. Richard Fabsitz, Dr. Cashell Jaquish, Dr. Teri Manolio and Ms. Lisa O'Neill.

NHGRI Project Office

Bethesda, MD--Dr. Elizabeth Thomson.

Dr. Jean MacCluer, Southwest Foundation for Biomedical Research, also contributed to the design of this study.

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Blood Cells Mol Dis. Author manuscript; available in PMC 2013 July 30.

Table 1

Characteristics of African-American HEIRS Study participants evaluated for *FPN1* Q248H[1]

		Serum Ferritin (μg/L)[2]		Transferrin Saturation, %		Initial Screening Self-Report		
Group	**Mean Age**	**Mean**	**Range**	**Mean**	**Range**	**Arthritis**	**Diabetes**	**Liver Disease**
All Controls								
(N = 210)	49 ± 15	68	7.5-287	24	4-68	36.1%	17.4%	0.5%
Men								
(N = 60)	50 ± 14	131	36-287	27	13-42	28.8%	22.6%	1.7%
Women								
(N = 150)	48 ± 15	53	7.5-193	23	4-68	38.8%	15.3%	0.0%
All Cases								
(N = 222)	52 ± 13	707	201-6,450	61	30-100	36.9%	20.4%	12.8%
Men								
(N = 106)	51 ± 11	812	304-6,450	65	35-100	28.6%	14.4%	11.1%
Women								
(N = 116)	54 ± 14	623	201-4,517	57	30-99	43.9%	25.5%	14.3%

[1]Of 432 participants in the present study, 428 (98.6%) were recruited from Field Centers in Washington, D.C. (121 men, 220 women) and Birmingham, Alabama (42 men, 45 women). The *FPN1* Q248 allele frequencies in these respective areas did not differ significantly (0.0411 vs. 0.0575, respectively; p = 0.348, X^2 test).

[2]Computations of mean serum ferritin were made using natural log-transformed data; tabulated results were re-transformed as antilogs.

Table 2

FPN1 Q248H genotypes in African-American participants in the HEIRS Study

***FPN1* Genotype**	**wt/wt**	**Q248H/wt**	**Q248H/Q248H**	**Total**	**P-value[1]**	**OR**	**95%CI**
All Cases	198	23	1	222			
All Controls	196	14	0	210			
Total	394	37	1	432	0.257	1.52	0.74-3.11
Men Only							
Cases	88	17	1	106			
Controls	57	3	0	60	0.047	3.64	1.02-13.01
Women Only							
Cases	110	6	0	116			
Controls	139	11	0	150	0.645	1.03	0.28-2.22

[1] Adjusted logistic-regression p-value, odds ratio (OR), and 95% confidence intervals.

Table 3

Self-reports of liver disease, *FPN1* Q248H, and serum ferritin levels African-American participants in the HEIRS Study[1]

Subjects	No Q248H	Q248H (heterozygote or homozygote)
Reported having liver disease		
Men	673 μg/L (110-4,640 μg/L) [11]	[0]
Women	636 μg/L (225-2,000 μg/L) [15]	[0]
Reported not having liver disease		
Men	366 μg/L (36-6,450 μg/L) [113]	647 μg/L (86-5,660 μg/L) [19]
Women	138 μg/L (7-4,703 μg/L) [211]	120 μg/L (7-2,080 μg/L) [17]

[1] Data are presented as mean serum ferritin concentration at initial screening (range) [number of subjects]. Computations of mean serum ferritin were made using natural log-transformed data; tabulated results were re-transformed as antilogs. None of the 26 participants who reported that they had liver disease had Q248H; this proportion did not differ significantly from the proportion of the participants who reported that they did not have a previous diagnosis of liver disease who had Q248H (0% vs. 10.0%, respectively; P = 0.0716, Fisher's exact test). Among participants who reported that they did not have a previous diagnosis of liver disease, the proportion of men with Q248H was sigificantly higher than the proportion of women with Q248H (14.4% vs. 7.5%, respectively; P = 0.0345, X^2 test).