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Heme carrier protein 1 (HCP1) genetic variants in the Hemochromatosis and Iron Overload Screening (HEIRS) Study participants

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Abstract

Heme carrier protein 1 (HCP1) has been identified as a possible heme carrier by *in vitro* analysis. To determine the association of mutations within the *HCP1* gene with iron phenotypes, we examined the entire coding region of the *HCP1* gene in 788 US and Canadian participants selected from the Hemochromatosis and Iron Overload Screening (HEIRS) Study using denaturing high-performance liquid chromatography. We sequenced the exon and flanking intronic regions if variants were detected. We tested 298 non-C282Y homozygotes from four racial/ethnic backgrounds (White, Black, Asian, and Hispanic) selected because they had high serum ferritin (SF) and transferrin saturations (TS). As controls, we chose 300 other random participants of the same racial/ethnic backgrounds from the same geographic locations. From the 333 HEIRS Study C282Y homozygotes, we selected 75 based on high SF and TS, 75 based on low SF and TS; 75 were selected randomly as controls. Thirty-five of the randomly selected C282Y homozygotes were also included in the high and the low SF and TS groups due to numerical limitations. We identified eight different *HCP1* genetic variants; each occurred in a heterozygous state. Except one, each was found in a single HEIRS Study participant. Thus, *HCP1* variants are infrequent in the populations that we tested. Five HEIRS Study participants had non-synonymous, coding region *HCP1* variants. Each of these five had TS above the 84th gender- and ethnic/racial group-specific percentile (TS percentiles: 84.7, 91.3, 97.9, 99.5, and 99.9).

INTRODUCTION

Increased iron absorption is a primary defect in hereditary hemochromatosis. The common mutation C282Y of the *HFE* gene on chromosome 6p occurs almost exclusively in Whites of European descent. Homozygosity for the C282Y mutation of the *HFE* gene on chromosome 6p accounts for most cases of hereditary hemochromatosis in this group [1;2]. H63D is another common *HFE* polymorphism that is found in most race/ethnicity groups. Iron overload occurs in some persons who are compound heterozygotes for C282Y and H63D and rarely in individuals without either H63D or C282Y mutations [1;2]. Other genes or mutations within *HFE*, could account for the marked variability in iron overload among *HFE* C282Y homozygotes [2–4;4–12]. No gene or mutation that accounts for a significant proportion of primary iron overload cases in non-Whites has been reported.

Most dietary iron occurs as either inorganic or heme moieties. Divalent metal ion transporter 1 (DMT1) facilitates the uptake of dietary non-heme iron across the microvillous membranes of absorptive enterocytes, but the transport system for heme iron absorption has remained

elusive. Recently, Shayeghi and colleagues [13] reported discovery of a gene they called heme carrier protein 1 (*HCP1*; MGC9564) whose expression appeared to facilitate heme absorption in the duodenal brush border membrane. *HCP1* expression appears to be regulated by a post-translational mechanism that responds to changes in body iron stores. Andrews suggested that HCP1 might be a major mediator of heme iron absorption by intestinal enterocytes, and that mutations within *HCP1* may influence body iron stores, either independently or as a modifier of clinical expression in individuals with other genetic configurations such as *HFE* C282Y homozygosity [14].

To test whether mutations in the *HCP1* gene influence serum iron measures either in those without *HFE* mutations or in *HFE* C282Y homozygotes, we used denaturing high-performance liquid chromatography (DHPLC) to screen the entire coding region of *HCP1* for mutations in 788 participants from the Hemochromatosis and Iron Overload Screening (HEIRS) Study [2; 7].

METHODS

Study Subjects

All subjects were participants in the Hemochromatosis and Iron Overload Screening (HEIRS) Study. A detailed description of this study's design and its 101,168 participants' general characteristics have been previously reported [4;15]. Each participant was classified into one of seven separate racial/ethnic groups based on self-report: White, Black, Asian, Hispanic (regardless of race reported), Pacific Islanders, American Indian, and multiple/unknown race/ethnicity. We included only Whites, Blacks, Asians, and Hispanics in the present *HCP1* study, because the numbers of HEIRS Study subjects who reported Pacific Islander, Native American, or multiple/unknown racial/ethnic groups were relatively small. Blood samples were obtained from all HEIRS Study participants at initial screening without regard to fasting, for measurement of serum transferrin saturation (TS), serum ferritin (SF), and genotyping to detect the *HFE* missense mutations C282Y and H63D. The HEIRS Study participants tested for *HCP1* gene mutations were chosen to achieve equal numbers for each study group (Table 1) and to satisfy budgetary constraints.

Selection of non-*HFE* C282Y homozygotes for DHPLC testing

We selected 75 non-C282Y homozygotes with very high SF and TS who reported either White, Black, Asian, or Hispanic race/ethnicity (Table 1). For comparison and to estimate gene frequency of *HCP1* variants, we also randomly selected 75 "control" participants from each of these four racial/ethnic groups (Table 1).

High TS/SF, non-C282Y participants—We first converted all HEIRS Study participants' TS and SF levels into gender-specific and *HFE* C282Y and H63D genotype-specific percentile values. We selected as cases for DHPLC testing the 75 participants within each racial/ethnic group who had the highest percentile for either TS or SF because we had no *a priori* reason to know whether *HCP1* mutations would influence SF or TS more strongly. Thus, the high TS/SF case group included participants if they had either a very high TS percentile or a very high SF percentile.

Racial/ethnic groups "control" selection—We selected 75 participants at random from each of the racial/ethnic groups, but we constrained the number of controls selected from each field center to match each field center's fraction of subjects in the high TS/SF cases groups.

Selection of *HFE* C282Y homozygotes for DHPLC testing

High TS/SF and low TS/SF C282Y homozygotes—From the 333 *HFE* C282Y homozygotes, we selected 75 with the highest SF and TS and another 75 with the lowest SF and TS using aforementioned gender- and field center-specific percentile-based transformation of the participants' SF and TS concentrations, without regard to *HFE* genotype.

HFE C282Y homozygote controls—We selected a control group of 75 subjects from the 333 HEIRS Study *HFE* C282Y homozygotes randomly with no constraints on field center, gender, or race/ethnicity (Table 1). All those selected were non-Hispanic Whites. We did not exclude those chosen for either the high or low TS/SF C282Y homozygote case groups from also being selected for the random control group. Accordingly, 35 of the 75 participants selected to be random C282Y homozygote controls were simultaneously members of high and low TS/SF groups. Altogether, 190 different *HFE* C282Y homozygotes were screened for *HCP1* variants.

Genotyping

DNA for DHPLC screening was either isolated directly from EDTA anticoagulated blood using the Puregene System (Gentra Systems, Minneapolis, MN) or by whole genome amplification with the GenomiPhi™ kit (GE-Amersham Biosciences Corp., Piscataway, NJ) from deproteinized buffy coat that had been spotted onto FTA® paper (Whatman, Clifton, NJ).

Primers were designed using IDT OligoAnalyzer (http://www.idtdna.com-/analyzer/Applications/OligoAnalyzer/) or Primer 3 (http://www-genome.wi.mit.edu/cgi-bin/primer/primer3_www.cgi) (Table 2). Each 50 μL PCR reaction contained 50–150 ng DNA, 200 μmol/L of each deoxynucleotide triphosphate, 10 mmol/L Tris-hydrochloride buffer (pH 8.3), 50 mmol/L potassium chloride, 2.5 mmol/L magnesium chloride, 1.5 U of HotstarTaq® DNA polymerase (QIAGEN, Valencia, CA), and 10–30 pmol of each primer. The reaction mixtures were brought to 94 °C for 15 min followed by 40 cycles of 94 °C for 30 sec, 30 sec at the appropriate annealing temperatures (Table 2), and an extension at 72 °C for 40 sec, followed by a final extension at 72 °C for 5 min using the PTC-225 Peltier thermal cycler (MJ Research, Inc., Waltham, MA).

Mutation screening was performed on a Transgenomic WAVE® 3500 HT fragment analysis system equipped with a DNASep HT cartridge. Eight μL of PCR product was injected onto the column and eluted with a linear gradient of buffer A and buffer B obtained from the manufacturer (Transgenomic, Omaha, NE., USA). The eluted amplicons were detected at 260 nm with a deuterium lamp. All chromatograms were analyzed using WAVE Navigator™ software version 1.5.3. Testing of samples for the possible presence of homozygous mutations was done by adding a sequence-determined wild-type amplicon to the amplicons prior to injection.

All DNA samples that appeared to have a *HCP1* variant were subjected to DNA sequencing using the same PCR amplicons used for DHPLC screening. Prior to sequencing, the DHPLC amplicons were purified using the Qiaquick® gel extraction kit according to the manufacturer's instructions (Qiagen, Valencia CA.). PCR amplicons were sequenced in both directions using 3.2 pmole of the same PCR primers with Big-dye Terminator sequencing kits (Applied Biosystems, Foster City, CA, USA). All sequencing was performed at the University of Minnesota's Advanced Genetic Analysis Center.

RESULTS

DNA samples were obtained from 790 HEIRS Study participants: 190 C282Y homozygotes and 600 with other *HFE* genotypes. Two of the 790 DNA samples could not be amplified by PCR. Among the 788 samples subjected to DHLPC screening of the *HCP1* gene's entire coding and partial intronic regions, we detected nine samples with *HCP1* genetic variants (Table 3). Each was present in a heterozygous configuration. Each variant was confirmed by sequencing in both directions using the same PCR DHPLC primers. No *HCP1* variants were found in the *HFE* C282Y homozygote high TS/SF group (Table 4). One *HCP1* synonymous coding region genetic variant was found in the C282Y homozygote low TS/SF group, and two different missense mutations were found in the C282Y homozygote random control specimens (Table 4). All five participants with *HCP1* coding region missense mutations had high TS, expressed either in absolute terms or as racial/ethnic group-, gender-, age-, field center-, and *HFE* genotype-specific percentile values. Two of these five participants had SF below the median (20.9^{th} and 39.4^{th} percentiles).

DISCUSSION

HCP1 genetic variants were very uncommon in the HEIRS Study participants we evaluated. The prevalences of *HCP1* variants in each of the four major racial/ethnic groups were similar in participants selected for high SF/TS and randomly selected controls. Each of the five participants with non-synonymous *HCP1* variants had TS above the race-, gender-, field center-, and *HFE* C282Y and H63D genotype-adjusted TS median values (Table 4). Only three of these had SF above the adjusted SF median. The TS percentiles of those with non-synonymous, coding region variants suggest a possible *HCP1* genotype-phenotype relationship, because it seems unlikely that all participants with a non-synonymous *HCP1* variant would be in the upper half of the TS percentile distribution by chance alone. Nevertheless, the prevalence of *HCP1* variants in our study population was low, and a much larger study would be needed to determine any significant association of *HCP1* variants with iron phenotypes.

Qiu and colleagues [16] have more recently suggested a function for the HCP1 protein that is not related to heme or iron transport. They expressed a cloned *HCP1* in *Xenopus* oocytes and in human hepatoma cells and observed that the gene product acts as a proton-coupled folate transporter (PCFT). Although Qiu et al. [16] amended the name of this gene's product to PCFT/HCP1, *HCP1* is called *SLC46A1* in GeneBank. However, some of the original findings of Shayeghi and colleagues[13] seem unexplained if *HCP1* is solely a folate transporter. For example, PCFT/HCP1 localizes to the plasma membrane in response to iron deficiency and its messenger RNA is up-regulated with hypoxia [13;14].

Part of Qiu and colleagues's rationale for classifying the gene product of *HCP1* as a folate transporter was based on their finding that two members of a single family who had phenotypes of Hereditary Folate Malabsorption (HFM, OMIM 229050) and who were homozygous for an intron2/exon3 boundary mutation in *HCP1* (nm_080669.2 c.1082-1 G>A) [16]. Recently, Zhao and colleagues from the same research group described five more HFM patients with homozygosity or compound heterozygosity for six additional *PCFT/HCP1* mutations [17]. They found no evidence of iron abnormalities in these patients, although each was an infant at the time of the study. No information on the iron status of the *HFE* patients' parents was reported. Because iron overload is usually a progressive, late-onset condition, it would be interesting to study the parents of these HFM patients for evidence of any abnormal iron phenotypes. We did not detect any of the specific *PCFT/HCP1* mutations previously reported by Zhao and colleagues [17] in the 788 HEIRS subjects that we tested. The HEIRS Study did not include measurements of serum or red cell folate concentrations, so we are unable to

comment on whether the PCFT/HCP1 mutations identified in HEIRS participants affect folate absorption.

We conclude that *HCP1* variants are uncommon in US or Canadian Whites, Blacks, Hispanics, and Asians. Although no single *HCP1* variant was associated with high TS or SF in our study, a disproportionate fraction of participants with non-synonymous, coding region mutations appear to have high TS.

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Table 1

Characteristics of HEIRS Study participants studied by DHPLC.

Groups	Serum Ferritin (SF) (μg/L)				Transferrin Saturation (TS) (%)				HEIRS Population Pool
	Mean SF	SD		Range of SF	Mean TS	SD		Range of TS	
Asian (cases) n=73	1,000.7	952.6	215	6,506	69.7	16.2	46	99	
Asian (controls) n=75	246.3	202.0	8	970	32.2	10.8	6	63	n=13,130
Black (cases) n=75	1,602.7	1260.9	205	6,450	76.4	17.6	46	100	
Black (controls) n=75	146.7	129.0	7.5	589	24.9	10.8	6	56	n=27,224
Hispanics (cases) n=75	763.5	721.2	205	3,800	62.6	15.9	46	99	
Hispanics (controls) n=75	123.8	135.1	8	781	25.8	10.3	5	61	n=12,696
White (cases) n=75	1,081.8	1072.4	209	5,300	71.5	18.3	30	100	
White (controls) n=75	160.3	218.1	8	1,650	28.5	13.9	5	100	n=44,808
C282Y homo/high iron n=75	1,065.4	970.4	40	5,200	90.8	12.6	38	100	
C282Y homo/low iron n=75	171.7	217.5	8	840	41.3	19.0	4	91	n=333
C282Y homo/controls n=75	500.8	685.0	8	5,200	63.5	24.8	4	100	

Table 2

Primers for *HCP1* exons and PCR conditions

	Primers	PCR Tm	DMSO
Exon 1	Forward: CGCCGGACATTTAAGGAGGGA Reverse: TCCAGTTACCCGCCACTACCATTA	63°C	4%
Exon 2.1	Forward: CAGAGTGAGGAACCGCAC Reverse: TGCGACTGGAGCTGACATCTG	68°C	No
Exon 2.2	Forward: GTGGCCTTCTGGCTGCTA Reverse: GTCCCTGAGCCCACACAC	65°C	No
Exon 3	Forward: CCCCATTTTCCTGATGAGTG Reverse: GCAGCACTGGACATGAGG	62°C	No
Exon 4	Forward: CGCCCGCCGCCGCCCCAGAGTATGGTATTTAT Reverse: CATTATGAGCATGGGTTCAG	64°C	No
Exon 5	Forward: CCTGGATATTGTCCTCCAGC Reverse: GTCTAACGTATGGCAGGCAG	64°C	No

Table 3

Distribution of *HCP1* genetic variants in HEIRS Study participants

nm_080669.2 (*HCP1* mRNA)	**Racial/ethnic groups**							
	Asian		**African American**		**Hispanic**		**White**	
Position and variation (Codon Frame)	**High N=73***	**Random N=75**	**High N=75**	**Random N=75**	**High N=75**	**Random N=75**	**High N=75**	**Random N=75**
c.85 G>A (GTC > ATC) (p.V29I)	0	0	0	0	**1**	0	0	0
c.189 G>C (AGG > AGC) (p.R63S)	0	0	**1**	0	0	0	0	0
c.228+17 G>A (IVS1+17)	**1**	0	0	0	0	0	0	0
c.228+76 G>A (IVS1+76)	0	0	0	**1**	0	0	0	0
c.756 C>G (GTC > GTG) (p.V252V)	0	0	0	0	0	**1**	0	0
c.883 A>G (ACA > GCA) (p.T295A)	0	0	0	**1**	0	0	0	0
	C282Y homozygotes from the study population							
	With highest TS/SF		With lowest TS/SF		Random selected homozygotes			
	N=75		N=75		N=75			
c.294 C>A (CTC > CTA) (p.L98L)	0		**1**		0			
c.1366 C>T (CCC > TCC) (p.P456S)	0		0		**2**			

* Two samples had insufficient or poor quality DNA and could not be PCR amplified.

Table 4

Serum iron parameters in HEIRS Study participants with heterozygous *HCP1* variants.

HCP1 Variants	*HFE* C282Y status	Serum Ferritin (SF) (μg/L)	SF Percentile in HEIRS subgroups[1]	Transferrin Saturation (TS) (%)	TS Percentile in HEIRS subgroups[1]	Age group[2]	Gender[3]	Ethnic group	HEIRS subgroup
Non-synonymous									
R63S	C/C	**1380**	99.9	**96**	99.9	40s	F	Black	case
T295A	C/C	**589**	92.0	**56**	99.5	40s	M	Black	control
V29I	C/C	**368**	97.7	**51**	91.3	50s	F	Hispanic	case
P456S	Y/Y	250	20.9	**96**	97.9	50s	M	White	control
P456S	Y/Y	172	39.4	**59**	84.7	50s	F	White	control
Intronic									
c.228+17G>A	C/C	**1450**	99.3	**78**	99.8	60s	M	Asian	case
c.228+76G>A	C/C	192	82.9	25	58.3	50s	F	Black	control
Synonymous									
L98L	Y/Y	131	9.5	36	1.9	60s	M	White	case
V252V	C/C[4]	79	15.6	39	68.6	30s	M	Hispanic	control

[1] Age-, gender-, field center-, and *HFE* C282Y and H63D genotype-specific percentile for TS or SF within the HEIRS Study [4]

[2] To preserve HEIRS participants anonymity, the HEIRS Steering Committee and NHLBI Project Office allows only reporting of participants ages by decade, not specific ages.

[3] F=female; M=male

[4] This individual is *HFE* H63D heterozygous.