Relationship between gene expression of duodenal iron transporters and iron stores in hemochromatosis subjects

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Nelson JE, Mugford VR, Kilcourse E, Wang RS, Kowdley KV. Relationship between gene expression of duodenal iron transporters and iron stores in hemochromatosis subjects. Am J Physiol Gastrointest Liver Physiol 298: G57–G62, 2010. First published November 5, 2009; doi:10.1152/ajpgi.00175.2009.—To test the hypothesis that differences in duodenal iron absorption may explain the variable phenotypic expression among HFE C282Y homozygotes, we have compared relative gene expression of duodenal iron transporters among C282Y homozygotes [hereditary hemochromatosis (HH)] with and without iron overload. Duodenal biopsy samples were analyzed using real-time PCR for expression of DMT1, FPN1, DCYTB, and HEPH relative to GAPDH from 23 C282Y homozygotes, including 5 “nonexpressors” (serum ferritin < upper limit of normal and absence of phenotypic features of hemochromatosis) and 18 “expressors.” Four subjects of wild type for HFE mutations without iron overload or liver disease served as controls. There was a significant difference in expression of DMT1 (P = 0.03) and DMT1(IRE) (P = 0.0013) but not FPN1, DCYTB, or HEPH between groups. Expression of DMT1(IRE) was increased among HH subjects after phlebotomy compared with untreated (P = 0.006) and nonexpressor groups (P = 0.026). A positive relationship was observed among all HH subjects regardless of phenotype or treatment status between relative expression of FPN1 and DMT1 (r = 0.5858, P = 0.0021), FPN1, and DCYTB (r = 0.5554, P = 0.0040), FPN1 and HEPH (r = 0.5100, P = 0.0092), and DCYTB and HEPH (r = 0.5400, P = 0.0053). In summary, phlebotomy is associated with upregulation of DMT1(IRE) expression in HH subjects. HFE C282Y homozygotes without phenotypic expression do not have significantly decreased duodenal gene expression of iron transport genes compared with HH subjects with iron overload. There is coordinated regulation between duodenal expression of FPN1 and DMT1, FPN1 and DCYTB, and FPN1 and HEPH and also DCYTB and HEPH in HH subjects regardless of phenotype.

Iron homeostasis; duodenal iron transporters; hemochromatosis; HFE gene; DMT1; ferroportin; gene expression; iron absorption

HEREDITARY HEMOCROMATOSIS (HH), an autosomal recessive disorder characterized by progressive accumulation of hepatic iron is one of the most common inherited diseases among Caucasians with an estimated prevalence of 1 in 250 and a heterozygote carrier rate of 8–10% (1, 3, 18, 22). If left untreated, irreversible organ damage, cardiomyopathy, diabetes mellitus, cirrhosis, and hepatocellular carcinoma may occur in the fourth or fifth decade of life. Most cases of hemochromatosis are due to a homozygous missense mutation in the HFE gene that results in a single amino acid substitution from cysteine to tyrosine (C282Y). Rarely, mutations in other genes involved in iron metabolism such as TFR2, HIV, and HAMP cause forms of hemochromatosis of varying severity collectively known as “non-HFE hemochromatosis” (16). There is incomplete penetrance of the C282Y mutation, and some studies have suggested that the majority of C282Y homozygotes may not develop end-organ damage (2, 4, 5, 24). It has been suggested that mutations in one or more disease-modifying genes may contribute to the variable phenotypic expression of HH (13–15).

Mammalian iron homeostasis is maintained primarily via regulation of dietary iron absorption. Absorption of inorganic iron in the small intestine is increased in response to a decrease in body iron stores. This process begins with the reduction of dietary ferric iron (Fe3+) in the duodenal lumen to ferrous (Fe2+) iron at the apical membrane of the mature duodenal enterocyte and is thought to be facilitated by the ferrireductase duodenal cytochrome b (DCYTB). Iron is then transported into the duodenal enterocyte by the divalent metal transporter-1 (DMT1). Once inside the enterocyte, iron is either complexed to ferritin for storage or directly transported across the basolateral membrane by ferroportin (FPN1), oxidized back to Fe3+ by the ferroxidase hephaestin (HEPH), and then transported into the circulation by binding to the iron storage protein transferrin. Hepcidin, a small 25-amino acid circulating peptide, is now recognized to be the major regulator of iron absorption and exerts its effect by binding to and internalizing FPN1 into the enterocyte, resulting in reduced iron efflux from the enterocyte (8, 17).

Several large-scale population screening studies have shown that a substantial proportion of C282Y homozygotes may not have phenotypic expression of iron overload or evidence of end-organ damage. The variable phenotypic expression of the homozygous C282Y genotype has been attributed to possible disease-modifying genes that may modulate intestinal iron absorption. However, previous studies examining the expression levels of duodenal iron transport genes in HH subjects have found conflicting results (7, 10, 12, 19, 23, 27–29). In addition, earlier reports have combined gene expression data from pre- and postphlebotomy subjects (7, 19, 28, 29); most importantly, no previous studies have included a cohort of C282Y homozygotes without iron overload (“nonexpressors”). We hypothesized that downregulation of duodenal iron transporter gene expression may explain the variable phenotypic expression among HFE C282Y homozygotes. Thus the goal of the present study was to examine whether 1) the variable penetrance of the C282Y mutation could be explained by differences in duodenal iron transporter gene expression, 2) phlebotomy therapy would increase expression of duodenal iron transporter genes, and 3) there is coordinated regulation of DMT1 and FPN1 expression and other iron transport genes across different pheno-type groups.
METHODS

Subjects. Subjects identified from the Iron Overload and Endoscopy clinics at the University of Washington Medical Center who agreed to have duodenal biopsies obtained via endoscopy for the purpose of this study or at the time of a clinically indicated endoscopy were enrolled in the study. A total of 29 unique duodenal biopsy specimens were obtained from 27 subjects; two subjects had biopsies before and after phlebotomy therapy and were therefore included in both the untreated and posttreatment groups. Twenty-three subjects were C282Y homozygotes, and four persons without HFE mutations, iron overload, or liver disease served as control subjects. Eighteen of the 23 C282Y homozygotes had phenotypic hemochromatosis [i.e., one or more of the following; an initial serum ferritin > the upper limit of normal (ULN, i.e., men > 300 μg/l, women > 200 μg/l) or increased hepatic iron concentration (i.e., > 2,000 μg/g dry wt)]. Nine specimens were obtained from subjects prior to phlebotomy treatment (mean serum ferritin 1,535 μg/l), and 11 specimens were obtained from iron-depleted subjects following phlebotomy treatment (mean serum ferritin 57 μg/l). Five subjects had a serum ferritin level < ULN, did not exhibit any phenotypic features of hemochromatosis, and were classified as nonexpressers (mean serum ferritin 118 μg/l). This study was approved by the institutional review board at the University of Washington, and written, informed consent was obtained from all subjects.

Sample collection. Typically, five biopsy samples were obtained from the second portion of the duodenum by use of standard endoscopy forceps. Samples were immediately snap frozen in liquid nitrogen and stored at −70°C for subsequent RNA purification. Simultaneously, serum iron indexes were obtained for each patient, including serum iron, transferrin-iron saturation (TS), and serum ferritin through the local clinical laboratory.

RNA preparation and cDNA synthesis. Total RNA was extracted by using TRIzol reagent (Invitrogen) according to the manufacturer’s protocol. RNA was then digested with DNase I (Roche) at 37°C for 1 h prior to cDNA synthesis. RNAs were washed in ethanol, resuspended in RNase-free water, then run on a Bioanalyzer 2100 (Agilent Technologies) to determine RNA quality and concentration. RNA quality was estimated from electrophoresis gel images, electropherograms, and the RNA Integrity Number (RIN) algorithm (20); samples below a RIN value of 3.0 were deemed degraded and excluded. Reverse transcription reactions of 1.5 μg RNA were performed with a Transcriptor First Strand cDNA Synthesis Kit (Roche) in 20-μl volume by using oligo(dT) primers following the protocol provided with the kit. The reactions are incubated for 1 h at 55°C followed by 5 min at 85°C to inactivate the reverse transcriptase. The cDNAs were brought to 300 μl volume in water (~5 ng/μl of the original RNA) prior to gene expression analysis.

Quantitative real-time PCR. Applied Biosystems predesigned gene expression assays containing both primers and fluorescent TaqMan probes were used for all expression assays. Primers and probes are designed to span intronic sequences to avoid amplification of any contaminant genomic DNA. The housekeeping gene glyceraldehyde phosphate dehydrogenase (GAPDH) was used for normalization of quantita-

tive and qualitative RNA variation. ABI part numbers for the primer/probe sets are as follows: FPN1, Hs00205888_m1; DMT1, Hs00167206_m1; HEPH, Hs00207710_m1; DCYTB, Hs00227411_m1; GAPDH, Hs99999905_m1. Additionally we assessed DMT1 for iron response element (IRE) splice variants with two ABI custom-designed primer/probe sets. The first probe (IRE+) bound at the IRE site (nucleotides 1814–1818 GenBank accession no. AB004857). The second probe (IRE−) recognized the non-IRE-containing segment (nucleotides 1814–1820, GenBank accession no. AF064484).

Each 20-μl real-time PCR reaction contained 2.0 μl 10× PCR buffer without Mg2+, 2.8 μl 25 mM MgCl2 (3.5 mM final concentration), 0.4 μl ROX passive reference dye, 0.4 μl 10 mM dNTPs, 1.0 μl ABI primer/probe, and 0.16 μl (0.8 U) Fast Start Taq Polymerase (Roche), 8.24 μl H2O, and 5 μl of the cDNA template. All reactions were run in triplicate in 384-well plates on an ABI7900HT and, for inclusion in the data set, we required standard deviations of the triplicates to be <0.15 cycles. Additionally, we verified that the PCR efficiencies of the ABI assays were >95% and that the slopes of the linear portion of the amplification curves varied by <5%.

Statistical analysis. Descriptive statistics of continuous variables were calculated and expressed as medians and interquartile range (IQR; 25th percentile–75th percentile). Sex was expressed as a proportion and compared between groups via Fisher’s exact test. Statistical differences between continuous variables were assessed by the Kruskal-Wallis test followed by post hoc comparisons between groups using the Wilcoxon rank-sum tests in conjunction with the Holm step-down procedure to obtain P values adjusted for multiple comparisons. Duodenal levels of iron transport genes were expressed as a ratio of GAPDH and reported as median and IQR. Spearman rank correlation coefficients and linear regression analyses were performed to determine the relationship between DMT1, FPN1, DCYTB, and HEPH gene expression and the relationship of expression of these genes to serum ferritin and TS levels. All statistical analyses were performed with STATA 9.0 (College Station, TX) except the Wilcoxon rank-sum tests in conjunction with the Holm step-down procedure were performed with R statistical software (www.R-project.org).

RESULTS

Clinical and laboratory differences in the study population. Clinical characteristics and serum iron studies of each patient group are shown in Table 1. All HH subjects (i.e., nonexpressors, untreated, and postphlebotomy groups) were homozygous for the C282Y mutation. Control subjects were wild type for both the C282Y and H63D mutations. The majority of subjects were male (66%); however, four of five subjects in the nonexpressor group (80%) were female. There was no significant difference in the sex or age of the groups. There were significant differences in total iron binding capacity (P = 0.02), TS (P = 0.005), and serum ferritin (P = 0.0003). The nonexpressor group, lacking features of phenotypic hemochromatosis,

Table 1. Comparison of serum iron parameters between patient groups

<table>
<thead>
<tr>
<th>Variable</th>
<th>Control (n = 4)</th>
<th>Nonexpressor (n = 5)</th>
<th>Untreated (n = 9)</th>
<th>Postphlebotomy (n = 11)</th>
<th>P Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, yr</td>
<td>33 (26.5–54)</td>
<td>36 (28–46)</td>
<td>46 (35–52)</td>
<td>54 (48–59)</td>
<td>0.15</td>
</tr>
<tr>
<td>Male sex</td>
<td>3 (75)</td>
<td>1 (20)</td>
<td>7 (78)</td>
<td>8 (73)</td>
<td>0.15</td>
</tr>
<tr>
<td>Serum iron, μg/dl</td>
<td>118.5 (79–148)</td>
<td>201 (133–229)</td>
<td>229 (203–230)</td>
<td>107.5 (92–232)</td>
<td>0.06</td>
</tr>
<tr>
<td>TIBC, μg/dl</td>
<td>338.5 (309–352)</td>
<td>267 (253–286)</td>
<td>251 (249–305)</td>
<td>315 (281–428)</td>
<td>0.02</td>
</tr>
<tr>
<td>Transferrin saturation, %</td>
<td>33.5 (25–42)</td>
<td>84 (53–86)</td>
<td>92 (91–92)</td>
<td>36 (21–80)</td>
<td>0.005</td>
</tr>
<tr>
<td>Serum ferritin, μg/l</td>
<td>76.5 (20–194)</td>
<td>35 (43–126)</td>
<td>1,208 (824–1,399)</td>
<td>23 (14–55)</td>
<td>0.0003</td>
</tr>
</tbody>
</table>

Data are presented as the median (25th to 75th percentile) or n (%). Statistical significance was determined by Kruskal-Wallis test. “Nonexpressor” was defined as serum ferritin < upper limit of normal and absence of phenotypic features of hemochromatosis. TIBC, total iron binding capacity.

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had significantly lower TS ($P = 0.04$) and serum ferritin ($P = 0.003$) compared with the untreated group.

Expression of DMT1, FPN1, HEPH, and DCYTB. We measured the expression of four genes by real-time PCR: FPN1, DMT1, HEPH, and DCYTB. Expression was normalized to GAPDH mRNA levels. There was a significant difference in expression of DMT1 across groups ($P = 0.03$) with the highest expression levels in the phlebotomized HH group (Fig. 1A).

There were no significant differences in expression of FPN1, DCYTB, or HEPH between groups. To determine the role of the IRE in DMT1 expression, custom primer/probe sets were also created to differentiate the IRE bound or non-IRE splice variant. There was a significant difference between groups in relative expression of $DMT1(IRE)$ ($P = 0.0013$) but not the non-IRE splice variant ($P = 0.23$, data not shown). A significant increase in $DMT1(IRE)$ expression was observed in the postphlebotomy HH group compared with both nonexpressors (2.7-fold; $P = 0.026$) and untreated subjects (5.9-fold; $P = 0.006$). Median $DMT1(IRE)$ expression was significantly lower among the overall untreated HH group compared with nonexpressors (2.2-fold; $P = 0.045$) (Fig. 1B). Further examination of the $DMT1$ expression data from the untreated HH subjects appeared to suggest the presence of two subgroups: those with low $DMT1$ expression ($n = 4$) and those with high $DMT1$ expression ($n = 5$) (see Fig. 2). Supporting the existence of these two subgroups is the fact that subjects in the “low $DMT1$/FPN1” group had significantly lower median serum ferritin values [median 838.5 (IQR, 498–1208)] compared with the “high $DMT1$/FPN1” group [median 1399 (IQR, 824–3630)] ($P = 0.05$). When we divided the untreated group into the two subgroups and compared gene expression across all groups, there were significant differences in relative expression of $DMT1$ ($P = 0.0026$) (Fig. 2A), $FPN1$ ($P = 0.035$) (Fig. 2B), and DCYTB ($P = 0.041$) (data not shown). Expression of both $DMT1$ and $FPN1$ was lowest in the untreated “low $DMT1$” patient subgroup.

Relationship between $DMT1$, $FPN1$, $HEPH$, and $DCYTB$ gene expression and correlation to levels of serum iron parameters. Spearman rank correlation coefficients were calculated to investigate associations among $DMT1$, $FPN1$, $HEPH$, and $DCYTB$ gene expression. There was a strong positive relationship between the following genes in all groups except the iron-depleted phlebotomy-treated HH subjects (see Fig. 3): $DMT1$ and $FPN1$ expression (all: $r = 0.5980$, $P = 0.0006$; HH only: $r = 0.5854$, $P = 0.0021$), between $FPN1$ and $HEPH$ (all: $r = 0.4438$, $P = 0.0159$; HH only: $r = 0.5100$, $P = 0.0092$); between $DCYTB$ and $HEPH$ (all: $r = 0.5064$, $P = 0.0051$; HH only: $r = 0.5400$, $P = 0.0053$) and between $FPN1$ and $DCYTB$ (all: $r = 0.4778$, $P = 0.0088$; HH only: $r = 0.5554$, $P = 0.0040$)

We also investigated the relationship between $DMT1$ and $FPN1$ expression with serum ferritin and TS levels. There was no correlation between serum ferritin or TS levels and either total $DMT1$ or $DMT1(IRE)$ in any group. $FPN1$ expression was not significantly associated with level of TS in any group but was significantly associated with serum ferritin in untreated subjects ($r = 0.8214$, $P = 0.0234$, data not shown). Serum ferritin and TS were also positively correlated to each other in the overall HH cohort ($r = 0.6521$, $P = 0.0014$, data not shown).

**DISCUSSION**

The present study is, to our knowledge, the first to examine the duodenal gene expression of mucosal iron transporters among HH subjects with and without phenotypic expression. Early studies examining the expression of duodenal iron transport genes in HH subjects were limited by the failure to analyze treated and untreated HH subjects separately (7, 19, 28, 29). Our goal was to examine whether lack of phenotypic expres-
sion in C282Y homozygous HH subjects (i.e., the nonexpressor group) could be explained by decreased expression of duodenal iron transport genes *DMT1, FPN1, DCYTB*, and *HEPH*. We did not observe decreased expression of duodenal iron transporter genes in subjects without phenotypic HH (nonexpressors) compared with the overall group of untreated HH subjects. By contrast, we observed that duodenal *DMT1* and *FPN1* gene expression among “nonexpressors” was similar to controls and at an intermediate level between the untreated subjects with mild to moderate iron overload (low *DMT1/FPN1* group) and those with marked iron overload (high *DMT1/FPN1* group). Phlebotomized HH subjects had higher expression of *DMT1/DMT1(IRE)* compared with all other groups. There was no significant difference between HH groups in the expression of *HEPH* or *DCYTB*. Similar to previous reports we observed a strong positive relationship between the expression of *FPN1* and *DMT1* (23, 26, 29), *FPN1* and *DCYTB* (29), *FPN1* and *HEPH* (29), and *DCYTB* and *HEPH* (10, 29) in all HH groups that had not been treated by phlebotomy, suggesting the presence of coordinated regulation of these genes in the absence of phlebotomy therapy, which may be disrupted because of the demands of erythropoiesis after iron depletion.

The most interesting finding in the present study was the unexpected observation of two subgroups within untreated HH subjects with iron overload that were distinguished by statistically significant differences in levels of serum ferritin and *DMT1* and *FPN1* gene expression: a group with mild-moderate levels of iron overload based on serum ferritin levels <1,000 μg/l characterized by significant downregulation of *DMT1* and *FPN1* and a distinct group with markedly increased body iron stores and significantly increased duodenal *DMT1* and *FPN1* expression. On the basis of this observation, we propose the novel hypothesis for the variable phenotype of *HFE*-associated HH. We speculate that among nonexpressors, there is compensatory increase in hepcidin expression early in life that allows the maintenance of a “normal setpoint” in the level of expression of duodenal iron transport genes to levels similar to normal individuals. However, among those who have phenotypic expression of HH, downregulation of *DMT1* and *FPN1* (possibly via induction of hepcidin expression in the liver) is accomplished at some point, allowing maintenance of body iron stores at a mild-moderate level (i.e., low *DMT1/FPN1* group). This compensatory response would be absent in those who develop progressive and continued iron overload and is due to failure to downregulate *DMT1* and *FPN1* in response to body iron stores (i.e., high *DMT1/FPN1* group). The differences between the low *DMT1/FPN1* and high *DMT1/FPN1* groups could be due to genetic or environmental factors such as the presence of modifying genes or excess alcohol consumption, which was recently shown to downregulate hepcidin production in the liver (11).

Several other findings in our study extended and clarified findings of previously published reports on gene expression of duodenal iron transporters. One previous study also found that levels of *DMT1* expression but not *FPN1* were significantly increased in postphlebotomized HH subjects compared with untreated HH subjects or control subjects (12); cumulatively, these data corroborate early studies showing that duodenal iron uptake is increased in response to increased erythropoiesis after phlebotomy (21, 25). Recent studies of gene expression of duodenal iron transport genes in untreated HH subjects have found variable results; one group reported significantly increased expression of *DMT1* and *FPN1* between untreated HH subjects and controls (26), whereas others failed to observe a difference between untreated HH subjects and normal subjects (23). We speculate that these discordant findings may have been because of variable numbers of subjects with mild-moderate (“low” *DMT1/FPN1*) vs. marked iron overload (“high” *DMT1/FPN1*) within these studies, which may have led to divergent findings in the rate of duodenal expression of *DMT1* and *FPN1* among untreated HH subjects.

![Fig. 2. DMT1 mRNA levels (A) and ferroportin (FPN1) mRNA levels (B) in duodenal biopsy samples from untreated “high” and untreated “low” subgroups compared with nonexpressing and postphlebotomy hemochromatosis subjects and control subjects. Values are a ratio of the gene of interest to GAPDH cDNA. Median (horizontal lines), interquartile range (25th percentile-75th percentile) (boxes), and upper and lower adjacent values (vertical lines) are shown. Statistically significant differences were observed between groups (Kruskal-Wallis test; A, P = 0.0026; B, P = 0.035).](http://ajpgi.physiology.org/Downloadedfrom/10.2307/3175233)
It is possible, if not likely, that a combination of genetic and environmental differences between these untreated HH patient subgroups may underlie their ability to regulate iron transport genes in response to body iron stores, independently or by modulating hepcidin expression. In support of this hypothesis, Milet et al. (15) screened 592 C282Y homozygotes with known serum iron studies for single nucleotide polymorphisms (SNPs) in 10 genes involved in hepcidin regulation (including \textit{HAMP} and \textit{FPN1}). These authors identified an allele in the bone morphogenic protein 2 (BMP2) in 13% of their cohort that had significantly lower serum ferritin levels (47 and 87%) lower compared with the other two alleles at this locus in their cohort. They also found other SNPs in the \textit{HJV} and \textit{BMP4} genes that had additive effects with the \textit{BMP2} allele resulting in even lower mean serum ferritin levels. Thus it appears there are now several candidate genes that may modify disease expression in HH.

We recognize that the present study has several important limitations. We did not have detailed information about alcohol consumption in our HH subjects and, more importantly, did not have serum or urine for hepcidin measurements or DNA to identify the presence of polymorphisms in other iron-related genes, which could have suggested possible mechanisms for the differences in duodenal gene expression among the various HH groups. Our hypothesis would also have been strengthened if we had liver tissue for determination of hepcidin gene expression. Furthermore the sample size in the present study was relatively small increasing the risk of a type 2 statistical error. We encourage other investigators to reproduce our results in a larger sample and to study concomitant hepcidin gene expression in liver tissue to verify these results.

In summary, the present study shows that there is coordinated positive regulation of \textit{FPN1} with \textit{DMT1}, \textit{DCYT1}, and
**HEPH of DCYT B with HEPH among normal subjects as well as HH subjects regardless of phenotype. Iron depletion via phlebotomy treatment in HH subjects resulted in significantly increased DMT1(IRE) gene expression compared with untreated and nonexpressor HH subjects. HFE C282Y homozygotes without phenotypic expression do not have significantly decreased duodenal gene expression of iron transport genes compared with HH subjects with iron overload.

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