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Expression of ferroportin in hemochromatosis liver

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Abstract

Iron-regulated transporter protein 1 (IREG1 or ferroportin 1) is a transmembrane iron transporter that has been described in macrophages and hepatocytes. Ferroportin mutations have been described to result in hepatic iron overload in human pedigrees. The role of hepatic ferroportin in the pathogenesis of C282Y-linked hemochromatosis has not been clearly established. The objective was to study the expression of ferroportin mRNA and protein in C282Y-linked hemochromatosis liver and in controls. Human liver biopsies were stained with an anti-ferroportin antibody and quantitation of ferroportin at 62 kDa was done by Western blotting. mRNA was studied by real time RT-PCR. Ferroportin protein expression was increased in C282Y homozygotes ($n = 23$) compared to wild-type patients ($n = 37$) ($P < 0.003$). There was no significant correlation between ferroportin protein or mRNA expression ($n = 25$) and liver iron concentration or serum ferritin. Immunohistochemical staining demonstrated ferroportin in hepatocytes and macrophages. In conclusion, ferroportin protein is increased in iron-loaded hemochromatosis liver. The increase in ferroportin protein without an increase in mRNA is consistent with iron-mediated translational regulation through the 5′IRE in the mRNA.

Introduction

Since the discovery of the *HFE* gene in 1996 [1], it has become increasingly apparent that there are more iron-related proteins involved in the pathogenesis of hemochromatosis than the HFE protein. The HFE protein has been demonstrated to have interactions with the transferrin receptor, hepcidin, beta-2-microglobulin, and divalent metal transporter 1 (DMT1) leading to an increase in intestinal absorption of iron from the duodenum [2–4]. However, the export of iron out of cells has also been an area of interest in hemochromatosis since ferrokinetic studies have suggested increased transport of iron across the basolateral membrane of intestinal cells [5]. Ferroportin or iron-regulated transporter protein (IREG1) was described by McKie et al. [6] to be an iron transport protein involved in the basolateral transfer of iron to the circulation. The 5′UTR of the ferroportin mRNA contains a functional iron-responsive element (IRE). Ferroportin expression has been demonstrated to be increased in mouse liver after iron loading by dextran injections [7]. Recently, two human pedigrees have been described with familial iron overload related to two different mutations in the ferroportin gene [8,9]. This was interpreted as an autosomal dominant form of hemochromatosis. The goal of this study was to determine if ferroportin protein expression is altered in response to hepatic iron overload. In this study, the expression of ferroportin is described in human hemochromatosis liver in patients who are homozygous for the C282Y mutation of the *HFE* gene.

Methods

Hemochromatosis patients were referred for abnormal iron studies or symptoms and underwent liver biopsy at the time of diagnosis because of abnormal liver enzymes or suspected liver dysfunction. All studies were done prior to phlebotomy therapy. Control patients were patients that were referred for liver biopsy for other indications (steato-

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sis, hepatitis C, hepatitis B, primary biliary cirrhosis, autoimmune hepatitis, and alcoholic liver disease) and exhibited normal liver iron concentration. Control patients were non-C282Y homozygotes and included H63D homozygotes ($n = 3$) and H63D heterozygotes ($n = 10$), C282Y heterozygotes ($n = 5$), and wild type ($n = 37$). A subgroup of control patients was examined that had an elevated hepatic iron concentration (>35 μmol/g dry weight) and were not C282Y homozygotes ($n = 14$). The most severe cases of secondary iron overload were two patients with beta-thalassemia and a hemodialysis patient with hepatitis B and 12 years of parenteral iron therapy. Other cases included alcoholic siderosis, hepatitis C, and a H63D homozygote with mild iron overload.

Genotyping was done by using primers that excluded the 5569A polymorphism of the *HFE* gene [10]. Hepatic iron concentration was measured by using atomic absorption spectrophotometry as previously described [11]. This project was approved by the Human Ethics Committee of the University of Western Ontario.

RNA extraction

RNA was extracted from biopsy samples using TRIzol reagent (Invitrogen Inc., Canada). Chloroform was used to separate the solution into aqueous and organic phases. RNA from the aqueous phase was precipitated with isopropyl alcohol and suspended in DEPC-treated water. RNA was quantified by measuring UV absorbance at 260 nm. Purity of the samples was assessed by calculating OD 260:280 nm. RNA was not available on all samples.

First strand cDNA synthesis

Five micrograms of total RNA was used for cDNA synthesis by employing Superscript-II system (Invitrogen Inc.). Oligo(dT) primers were added to total RNA and the samples were denatured at 70°C. MMLV-reverse transcriptase was added to the samples, which were further incubated at 42°C for 50 min. The reaction was stopped by incubating the samples at 70°C for 15 min.

Real time RT-PCR

cDNA from biopsy samples was subjected to real-time quantitative RT-PCR in LightCycler (Roche Diagnostics Canada, Quebec, Canada) using SYBR Green I detection platform. PCR was performed in a total reaction volume of 20 μL each and run in duplicates in microcapillaries (Roche Diagnostics Canada). The PCR reaction mixture consisted of 2.0 μL of DNA FastStart SYBR Green Master Mix (Roche Diagnostics Canada), 1.6 μL of 25 mM $MgCl_2$, 1.0 μL of each forward and reverse 10 μM primers (Table 1), 13.4 μL of H_2O, and 1.0 μL of cDNA. Thermal cycling parameters are given in Table 1. During optimization of ferroportin and 18S rRNA PCR, melting curve analysis was used to determine melting temperature (T_m) of specific amplification products and primer-dimers. The T_m values of specific ferroportin and 18S rRNA products were used to add an additional step (signal acquisition step; Table 1) following the extension phase for subsequent PCR reactions. This additional step (2–3°C below the T_m of amplification products) allowed for signal acquisition from target sequences without interference from primer-dimers and nonspecific amplification products (Fig. 1).

Table 1
Primer sequences and PCR parameters

Primer sequences	PCR temperature profile[a] (temperature, time)
Ferroportin	Denaturation 95°C,0 s
5′-CAGTTAACCAACATCTTAGC-3′	Annealing 49°C, 3 s
5′-AAGCTCATGGATGTTAGAG-3′	Extension 72°C, 13 s
	Signal acquisition 80°C, 1 s
18S rRNA [20]	Denaturation 95°C, 0s
5′-GTAACCCGTTGAACCCCATT-3′	Annealing 55°C, 5 s
5′-CCATCCAATCGGTAGTAGCG-3′	Extension 72°C, 9 s
	Signal acquisition 80°C, 1 s

[a] Initial denaturation at 95°C for 10 min. Ramp rate for all steps was 20°C/s.

Ferroportin mRNA expression was determined by using a standard curve method. Standard curves were constructed by using serial dilutions of total RNA. The C_p (Crossing Point; point at which fluorescent signal is statistically different from baseline signal) was plotted against the template amount. C_p value obtained for samples was used to compute template concentration from the standard curves. The samples were then normalized against 18S rRNA to account for differences in template amount and reverse transcription efficiency.

Protein purification and Western blotting

Liver tissues were homogenized in complete RIPA buffer [NaCl 0.877g, deoxycholate 1 g, 1 M Tris-HCl (pH 7.5) 5 mL, Triton X-100 1 mL, and 10% SDS 1 mL; volume adjusted to 100 mL using ddH_2O) and protease inhibitor. Following purification, total proteins were quantified by using the BCA protein assay kit (Pierce Endogen, Rockford, IL).

Total proteins were resolved by 10% sodium dodecyl sulfate–polyacrylamide gel electrophoresis and analysed by Western blotting. A polyclonal ferroportin antibody was produced in rabbits against a synthetic peptide and affinity-purified (ResGen, Invitrogen Corporation, Huntsville, AL). An antibody against this peptide has been previously characterized and shown to be specific for ferroportin [6]. Western blots were produced as previously described [6]. β-Actin was used for loading control. Secondary goat anti-rabbit antibody conjugated with horseradish peroxidase (Bio-Rad Laboratories, Hercules, CA) was used to produce signals from the chemiluminescent substrate, Luminol (Amersham

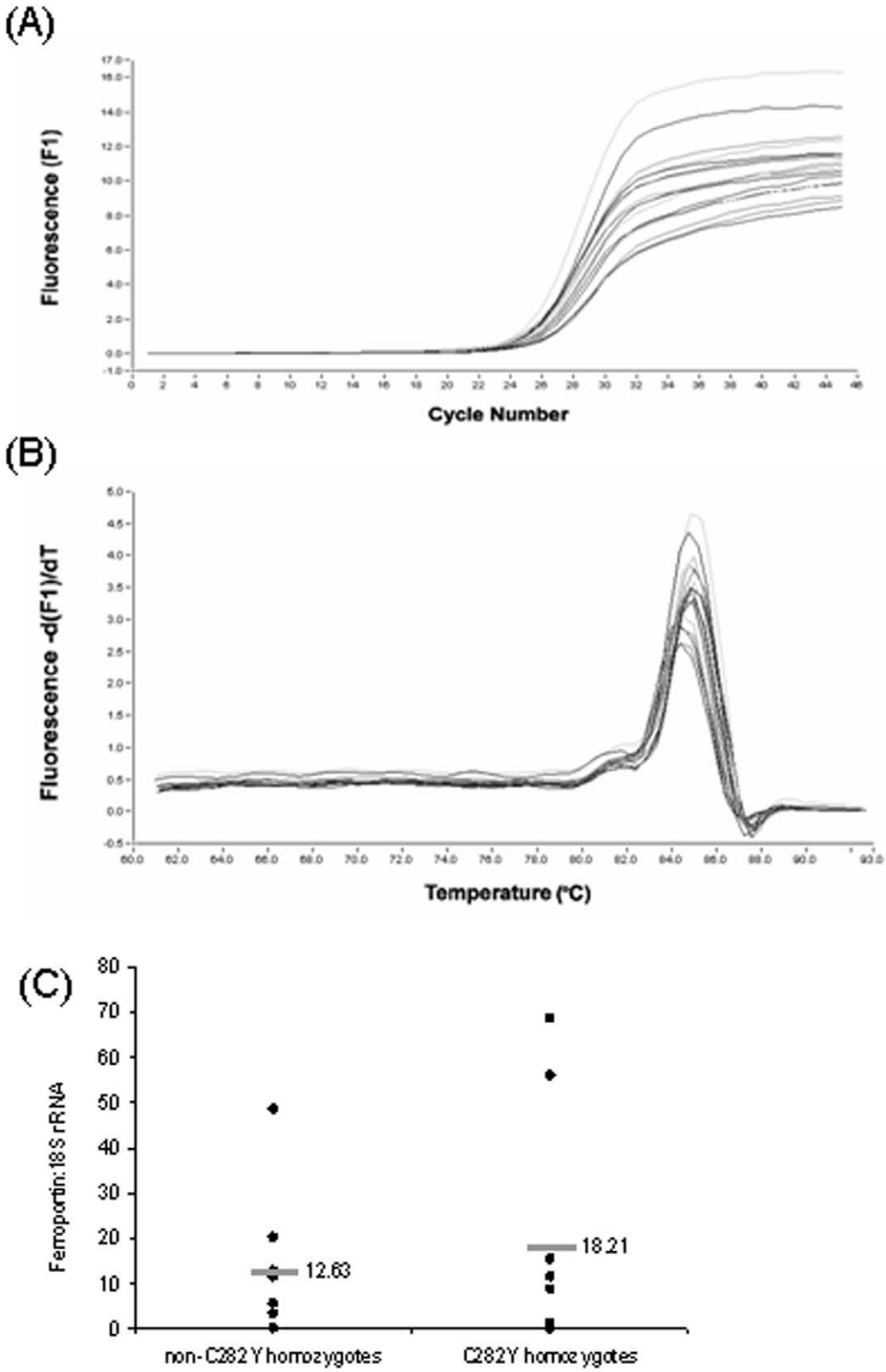

Fig. 1. Quantification of ferroportin mRNA transcripts by real time RT-PCR showing (a) PCR amplification plot of ferroportin, (b) melting curve analysis used to determine specificity of PCR reactions, and (c) quantification of ferroportin mRNA in C282Y homozygotes. Ferroportin mRNA levels were expressed as ratio of target gene to 18S rRNA.

Table 2a
Ferroportin protein expression

Study group	*n*	Age/gender	Ferritin	Liver iron concentration	Ferroportin protein
C282Y homozygotes	23	49 yr (33–78) 19 men, 4 women	230 μg/L (362–6443)	292 μmol/g (58–632)	2.5[b] (1.4–3.9)
All other non-C282Y homozyogotes	55	49 yr (33–75) 36 men, 19 women	798 μg/L (8–4080)	36 μmol/g (1.5–365)	2.1[b] (1.4–4.2)
Wild-type patients	37	48 yr	754 μg/L (8–4080)	46 μmol/g (2.6–365)	2.0[b] (1.6–3.1)

[a] Data are expressed as mean (range). Liver iron concentration in μmol/g dry weight units.
[b] Ferroportin protein expression was significantly greater between C282Y homozygotes and all other non-C282Y homozygotes ($P < 0.02$, *t* test) and wild-type patients ($P < 0.003$, *t* test).

Pharmacia Biotechnology, Buckinghamshire, UK). The signals were quantified by densitometry and expressed as arbitrary units per milligram of protein.

Immunohistochemical staining

Immunohistochemical staining of liver was done by using a primary antibody concentration of 1:100 (1 h incubation) and an Avidin-biotin peroxidase (ABC) method. Negative controls included incubation with PBS without the primary antibody. Specificity of the antibody was confirmed by blocking the sections in 10% horse serum.

Results

A comparison of the study groups is shown in Table 2. There was a significant increase in ferroportin protein expression in C282Y homozygotes compared to all nonhomozygotes (two-tailed *t* test, $P < 0.02$) and compared to wild-type patients ($P < 0.003$). We have used a sensitive real-time PCR-based assay to investigate ferroportin mRNA transcripts (Fig. 1). There was no significant difference in ferroportin mRNA between C282Y homozygotes and nonhomozygotes ($P = 0.58$; Fig. 1). Correlation coefficients were not significant between liver iron concentration and ferroportin protein and mRNA, or between ferroportin protein and mRNA. Ferroportin protein expression was not significantly increased in 14 non-C282Y homozygotes with an elevated liver iron concentration (mean 99 μmoL/g; range 36–365) compared to the other control patients.

The quantitation of ferroportin in liver tissue by Western blotting in relationship to liver iron concentration is shown in Fig. 2.

The relationship between hepatic iron concentration, *HFE* genotype, and ferroportin protein expression is shown in Fig. 3.

Immunohistochemical staining demonstrated the distribution of ferroportin that was increased in iron-loaded hepatocytes and Kupffer cells (Fig. 4).

Discussion

Since C282Y hemochromatosis has a wide range of clinical expression, there has been interest in the effects of comodifying genes and iron-related proteins. It is increasingly apparent that a cascade of events involving a number of proteins occurs both in normal iron metabolism and in both iron deficiency and iron overload. In this study we have studied the relationship of ferroportin protein and mRNA to hepatic iron overload in C282Y-linked hemochromatosis. In C282Y hemochromatosis, our current understanding of increased intestinal iron absorption has emphasized the roles of *HFE*, hepcidin, transferrin receptor, and DMT1. It has been assumed that the hepatic iron overload is an upstream consequence of the efflux of iron into the portal circulation, but the iron uptake pathways on the hepatocyte in hemochromatosis have not been clearly established. Transferrin receptors have been described to be downregulated on hepatocytes in hemochromatosis [12] and other non-transferrin-mediated pathways have been studied. DMT1 and hepcidin may also play a role in the intracellular metabolism of hepatic iron [13,14]. The excretion of iron by hepatocytes into bile has been reported to be increased in hemochromatosis[15] and ferroportin may be involved in the transport process. In the setting of hepatic iron overload, an upregulation of ferroportin that contains an iron-responsive element could be a cellular defense mechanism to decrease the toxic effects of excess iron. In the setting of a ferroportin

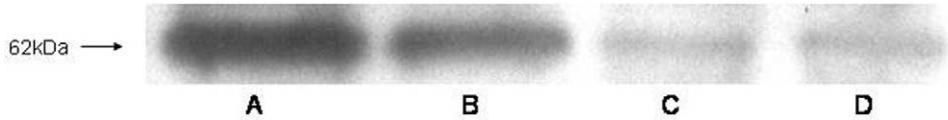

Fig. 2. Western blot from C282Y homozygotes (A and B) and nonhomozygotes (C and D) showing increased ferroportin protein expression in the former at 62 kDa.

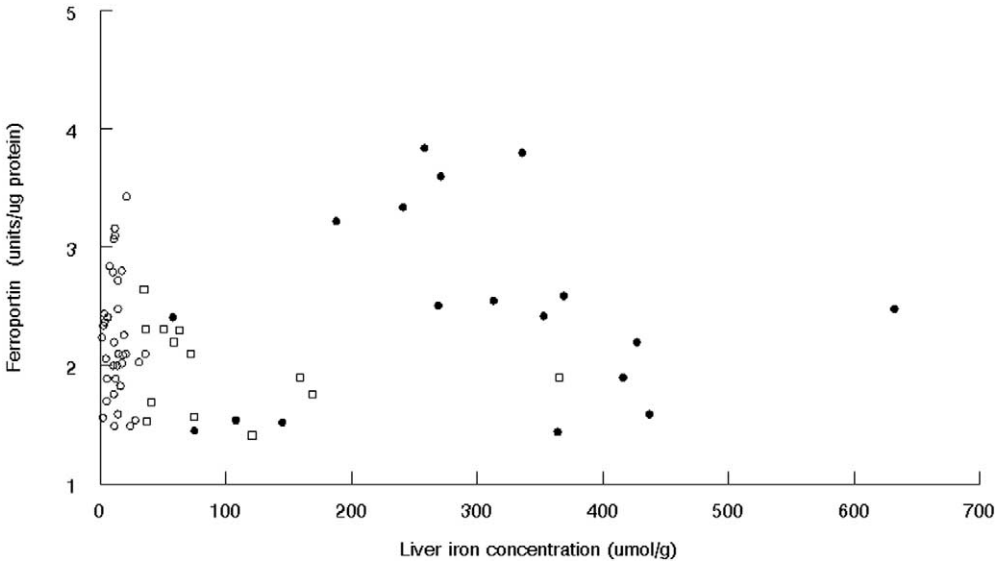

Fig. 3. The relationship of liver iron concentration and ferroportin expression in iron-loaded C282Y homozygotes (●), H63D homozygotes (□), H63D heterozygotes (Δ), C282Y heterozygotes (◊), and wild-type patients (○).

mutation, hepatic iron excretion may be impaired, which results in a new clinicopathological syndrome of iron overload that has recently been described in Italian and Dutch pedigrees [8,16].

Our study has demonstrated a modest increase in ferroportin protein in iron-loaded hemochromatosis liver. However, there is a significant overlap with other liver disease patients. One possibility is that inflammation

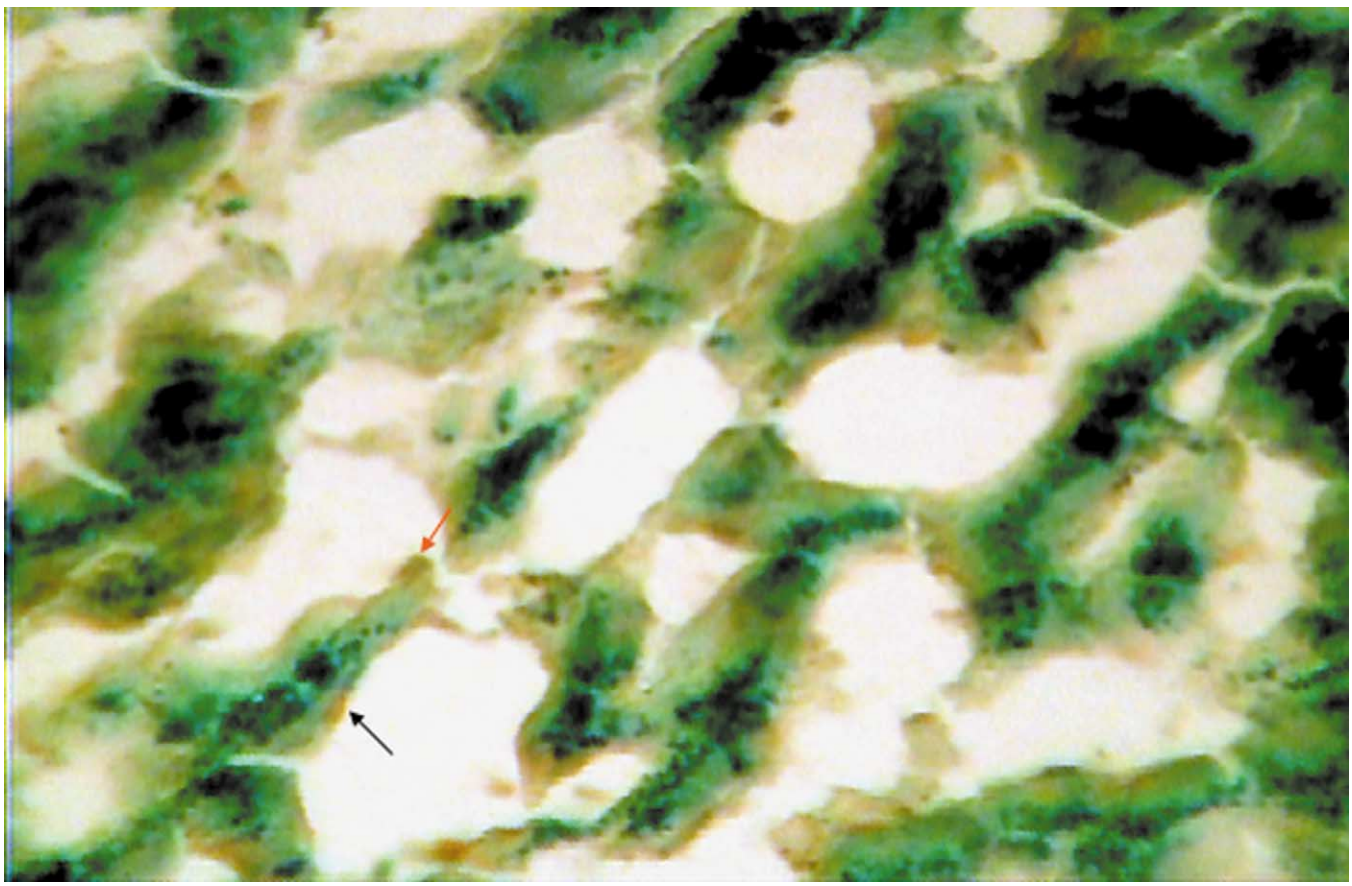

Fig. 4. Photomicrograph of liver from a known C282Y homozygote showing increased iron deposition (blue) in association with ferroportin expression (brown stain) in hepatocytes (black arrow) and Kupffer cells (red arrow) (dual immunoperoxidase and Perl's Prussian Blue stain; original magnification ×400).

(hepatitis C, and steatohepatitis) could increase expression of ferroportin protein in the control patients independent of iron. Similar observations on the effects of cellular activation have been made for transferrin receptors [17]. Ferroportin has significant expression in macrophages that are paradoxically not iron loaded in hemochromatosis. Therefore, other iron conditions such as transfusional iron overload may result in more expression of ferroportin than hemochromatosis patients. In this study there were several cases with significant secondary iron overload that showed elevations of both ferrportin protein and mRNA. Our studies have not demonstrated an increase in mRNA in iron-loaded hemochromatosis liver. The increase in ferroportin protein without an increase in mRNA is consistent with iron-mediated translational regulation through the 5′IRE in the mRNA. This is similar to ferritin translation by iron. In contrast to our data, a previous study has shown an increase in liver ferroportin mRNA in hemochromatosis liver [18]. Variable techniques used to quantify ferroportin mRNA transcripts may be a partial explanation. Our data are, however, consistent with the data of Gehrke et al. [19], which demonstrate no upregulation of ferroportin mRNA levels in hemochromatosis using a PCR-based assay.

Acknowledgments

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