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Immunohistochemistry of HFE in the duodenum of C282Y homozygotes with antisera for recombinant HFE protein

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ABSTRACT

Background and Objectives. HFE is a class-I MHC related protein which carries the C282Y mutation in most patients with hereditary hemochromatosis, an iron overload disease. HFE protein is expected to have a relevant role in the regulation of duodenal iron absorption, and HFE protein was immunohistochemically identified in the crypt cells. The aim of the work was to analyze whether the C282Y mutation affects HFE accumulation in the duodenum.

Design and Methods. We developed antisera for the extracellular portion of recombinant human HFE protein expressed in _E. coli_. The antisera were specific for HFE protein and the C282Y mutant in immunoblotting, immunoprecipitation and immunocytochemistry experiments of transfected cells, and they did not cross react with HLA antigens in various analyses. The antisera gave positive results in the staining of paraffin-fixed sections of duodenal slices of subjects with hemochromatosis.

Results. The antisera stained evident supranuclear granules in all enterocytes of 7 C282Y homozygous subjects, and a dark area in the same region in 3 other C282Y homozygotes. Granular bodies were absent from the duodenal sections of 8 C282Y negative subjects, from 2 C282Y heterozygotes and 3 C282Y homozygotes, with or without hemochromatosis.

**_Interpretation and Conclusions._ The detection of HFE-protein in granular bodies in the enterocytes of the large majority (77%) of C282Y homozygotes and not in other subjects suggests that the mutation facilitates protein accumulation in the duodenum.
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Key words: hereditary hemochromatosis, iron metabolism, HFE, immunohistochemistry.

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Hereditary hemochromatosis (HHC) is a common autosomal recessive disorder characterized by an upregulated iron absorption which may lead to progressive iron deposition in parenchymal cells of liver, heart, joints and endocrine glands with toxic effects.[1,2] The candidate gene for HHC encodes the HFE protein (formerly known as HLA-H) resembling a major histocompatibility complex MHC class-I molecule.[3] The HFE Cys282→Tyr (C282Y) mutation is homozygous in 83-100% of HH subjects from the USA, North Europe and Australia[4-6] while it is less common in Italy (69%).[7] The clinical significance of a second mutation, H63D, has not been fully established, although a few HHC subjects were found to be compound heterozygotes for both mutations.[3]

The C282Y mutation results in the loss of a structural disulfide bond in the $\alpha 3$ domain of the protein, which prevents association with β_2-microglobulin (β_2m) and proper presentation to cell surfaces.[8,9] Transgenic mice HFE$^{-/-}$,[10] β_2m$^{-/-}$,[11] and homozygous for the C282Y mutation[12] show fast accumulation of iron in the parenchymal cells of liver similar to that occurring in HHC, however the severity of iron loading is higher in the HFE null mice than in the other two animal models.[10,12] *In vitro* studies have shown that HFE protein associates with the transferrin receptor (TfR) more tightly under neutral (pH 7.5) conditions than at acidic pH (pH 6) and that the binding reduces TfR affinity for Fe transferrin.[13,14] In transfected cells an association between HFE and TfR occurs in the endoplasmic reticulum/cis-Golgi compartment soon after synthesis[15] and reduces transferrin-mediated cellular iron uptake.[16,17] Caco-2 cells, which have morphologic and biochemical features of mature small intestine enterocytes, express normal HFE protein which is apparently upregulated by iron treatment.[18]

Northern blotting analyses indicated that mRNA HFE is expressed in various tissues with higher levels occurring in liver and intestine.[3] Antisera for the cytoplasmic C-terminal peptide allowed immunohistochemical identification of HFE in the apical plasma membrane of syncytiotrophoblasts of human placenta[19] and in the epithelial cells of the gastrointestinal tract with unique localization in the crypt cells of the small intestine,[20,21] while a monoclonal antibody for the renatured recombinant HFE protein identified the protein in Kuppfer cells, in liver and brain sinuosoidal cells, and in scattered epithelial cells in the crypts of both small and large intestine.[22] The immunohistochemical stains were generally weak, likely due to the low level of accumulation of the protein in tissues, and, remarkably, no data have been reported so far on the duodenal distribution of HFE protein in HHC. This distribution is relevant in order to understand the relationship between the HFE C282Y mutation and the upregulation of intestinal iron absorption typical of HHC. To this end we elicited antisera for recombinant HFE from *E. coli*, showed that they were specific for HFE protein and used them in the staining of duodenal sections of subjects with hemochromatosis.

Design and Methods

Cloning HFE cDNA

Human HFE cDNA was obtained by reverse transcriptase-polymerase chain reaction (RT-PCR) from mRNA extracted from human pulmonary cells. The cDNA was subcloned into the pCDNA3.1 vector (Invitrogen) for expression in mammalian cells fused to the myc-tag, producing the p3.1-HFE construct. The cDNA for domains $\alpha1$, $\alpha2$ and $\alpha3$ (residues 26-304) was subcloned into pET12b vector fused to a polyhistidine-tag, producing the pET-HFE construct. DNA sequencing confirmed that the encoded protein had the correct sequences.[3]

Recombinant HFE from E. coli

HFE protein was expressed in the transformed *E. coli* strain BL21(DE3)pLysS essentially as described elsewhere.[23] The insoluble fraction of cell homogenates was resuspended in 6 M guanidine HCl pH 8.0 and the protein purified by affinity chromatography on Ni-NTA agarose columns (Qiagen). The homogeneity of the purified protein was confirmed by SDS-PAGE and Coomassie blue staining. Protein concentration was determined by the BCA assay (Pierce) calibrated on bovine serum albumin.

Western blotting was performed as described elsewhere[23,24] and bound activity was revealed by ECL (Amersham). To assess cross-reactivity with HLA molecules we used the FlowPRA I Screening Test (One Lambda Inc., CA, USA) following the manufacturer's instructions. This test consists of beads coated with 30 different purified HLA Class I antigens designed to detect anti-HLA antibodies. The beads were incubated with the HFE antisera (dilution 1:50 or higher) and then with fluorescent labeled anti mouse-IgG antiserum and analyzed by flow cytometry. Anti HLA antibodies were used as positive controls.

Transfectant cells

HeLa cells were co-transfected with p3.1-HFE and pREP7-nβ_2m vectors[25] using the calcium phosphate method.[24] Cells were metabolically labeled for 2 h with 25 μCi/mL ^{35}S-methionine, lysed on the plate and total radioactivity of soluble proteins determined by trichloroacetic acid precipitation. The cytosolic lysates (2×10^6 cpm) were immunoprecipitated with either 4 μg of anti myc-tag antibody 9E10 (Sigma-Aldrich), with 1.5 μL of the anti-HFE antisera or with 2 μL of WS/32 antibody,[26] incubated with 30 μL of protein A-Sepharose (50%) and separated on 12% polyacrylamide SDS-PAGE. The gels were treated with enhancer (Amplify, Amersham) and exposed to autoradiography. Immunocytochemistry was performed on fixed and permeabilized transfectant cells, incubated with anti-HFE antiserum (1:200) or anti myc-tag antibody (2 μg/mL) followed by TRITC-labeled secondary antibody; the fluorescence stain was visualized by fluorescence microscopy.

Antibody production

Affinity purified HFE (50 μg) in 6M urea was mixed with complete Freund's adjuvant and injected subcutaneously. The mice were boosted at two-week intervals first with 50 μg of HFE in incomplete Freund's adjuvant, and then with HFE protein in saline. Alternatively, after the primary injection, some animals were boosted at two-week intervals with 50 μg of purified p3.1-HFE vector diluted in saline. Ascitic fluid production was induced in some mice by intraperitoneal injection of pristane.[27] Antibody titer was assessed by Western blotting.

Immunohistochemistry

Duodenal biopsies were endoscopically obtained, after informed consent, from subjects with no evidence of iron overload who underwent endoscopy for pyrosis and from patients with hemochromatosis, diagnosed according to standard criteria. These are: i) no known cause of iron overload, ii) an amount of iron removed to reach depletion higher than 5 g and 4 g for males and females, respectively, iii) liver iron concentration/age higher than 2, iv) histology with moderate to severe hepatocytic siderosis. Eight patients with hemochromatosis underwent endoscopy at diagnosis and ten after iron depletion therapy. Mutations of the HFE gene were analyzed as previously described.[28] Paraffin-embedded tissues were sliced, the sections deparaffinized in xylene and rinsed in absolute ethanol. Immunostaining was performed essentially as already described[29] using an avidin-immunoperoxidase technique with the reagent of the Vectastain Kit (Vector Laboratories). Endogenous peroxidase was blocked by incubation in 0.3% H_2O_2. The slides were hydrated in 20 mM phosphate buffer, 1% bovine serum albumin. The slides were washed in the same buffer, and incubated for 30 min with secondary, biotinylated anti-mouse immunoglobulin antiserum (Vector) diluted 30-fold and then with avidin-peroxidase complex. Enzyme activity was revealed with 3,3'-diaminobenzidine tetrahydrochloride (Sigma-Aldrich), the sections were counterstained with Mayers' hematoxylin (Sigma-Aldrich) and mounted.

Results

Recombinant HFE and antibody production

The extracellular portion of HFE protein fused to a His-tag was expressed in *E. coli* accounting for about 60% of the total insoluble proteins (Figure 1, lane 4). The ~39 kD protein was resolubilized in 6 M guani-

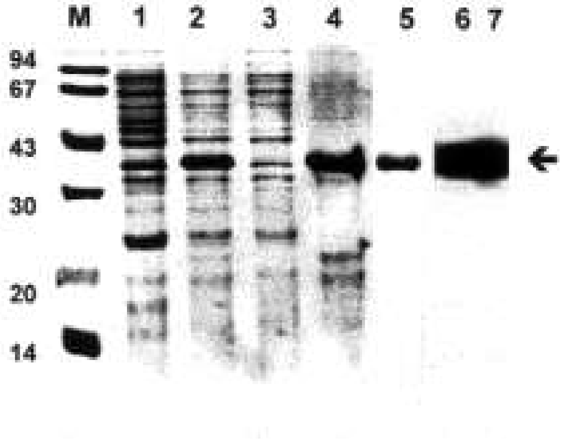

Figure 1. Recombinant HFE from *E. coli.* Over-expression of HFE in transformed BL21(DE3)pLys-S was induced by treatment with 1 mM IPTG for 3h. The cells were recovered, sonicated and insoluble proteins separated by centrifugation. The rHFE was resolubilized with 6 M guanidine HCl pH 8.0 and purified by affinity chromatography on an Ni-NTA agarose column. Lane 1: total cell homogenate of untransformed *E. coli*, lane 2: total homogenate of transformed cells, lane 3: soluble fraction, lane 4: insoluble fraction of the homogenates of transformed cells, lane 5: purified rHFE after affinity column. Lanes 6 and 7: Western blotting of the total cell homogenate and of purified rHFE, respectively, overlaid with anti-HFE antiserum (1:1000) and peroxidase labeled anti-mouse Ig. Lanes 1-5: Coomassie blue stain, lanes 6,7: ECL stain. M: molecular weight markers. The arrow points to rHFE.

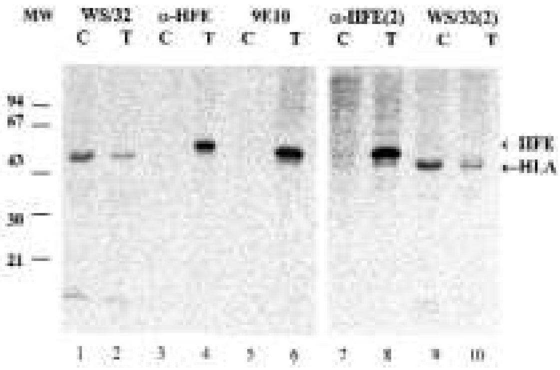

Figure 2. Immunoprecipitation of newly synthesized HFE. The cytosolic homogenates of ^{35}S-methionine metabolically labeled HeLa cells, either untransfected (lanes C) or transfected with HFE cDNA (lanes T) were immunoprecipitated with 2 µL of WS/32 for HLA-A, B and C (lanes 1,2), with 1.5 µL of anti-HFE antiserum (lane 3,4), or with 4 µg of anti-myc monoclonal antibody 9E10 (lanes 5,6). To test the lack of cross-reactivity between WS/32 and anti-HFE antibodies further, anti-HFE antibody was added to supernatants after WS/32 precipitation and the precipitates analyzed in lanes 7 and 8. Conversely, WS/32 antibody was added to the supernatant after anti-HFE precipitation, precipitated and analyzed in lanes 9 and 10. The immunoprecipitated proteins were separated on 12% SDS-PAGE and exposed to autoradiography. The arrow points to the bands attributed to the mature and tagged HFE protein and to HLA antigen. MW: molecular weight markers.

dine HCl and purified on an Ni-NTA agarose column obtaining about 8 mg of electrophoretically pure recombinant HFE (rHFE) per liter of cell culture (Figure 1, lane 5). Approaches to refold the HFE in the presence of purified recombinant β$_2$m, as described elsewhere[22] yielded low amounts of soluble protein which could not be characterized in detail. We, therefore, chose to use the denatured and purified HFE protein as the antigen to elicit antibodies. Mice were injected with the purified protein and boosted either with the protein or with p3.1-HFE vector encoding for the full HFE protein sequence. The antisera stained a single ~39 kD band in immunoblottings of purified rHFE and of total homogenates from transformed *E. coli* (Figure 1, lanes 6,7) at antisera dilutions of 1:1,000-2,000 using ECL development. In immunocytochemistry they decorated the permeabilized HeLa cells transfected with cDNA of HFE wild type or C282Y mutant with a similar morphology, which was analogous to that obtained with the 9E10 antibody specific for the myc-tag attached at the C-terminus of the recombinant proteins (not shown). Positive transfected cells reached 10-30% and the remaining negative ones did not show significant background. The cells were metabolically labeled with ^{35}S-Met and subjected to immunoprecipitation experiments. The HFE antisera precipitated a major band of ~49 kD attributed to the glycosylated form of HFE protein, which was absent from the non-transfected parent cells (Figure 2, lanes 3 and 4). A similar band was precipitated by the 9E10 anti-myc-tag antibody (Figure 2, lanes 5 and 6), while the anti HLA antibody WS/32 precipitated a band of faster mobility (~45 kD) from transfected and untransfected parent cells (Figure 2, lanes 1 and 2). The lack of cross-reactivity between the anti-HFE and WS/32 was confirmed by sequential immunoprecipitation experiments (Figure 2, lanes 7-10). The evidence that HFE antisera do not bind the HLA-A, B and C antigen recognized WS/32 antibody[26] supports their specificity for HFE protein. This was further proven by experiments using a FlowPRA I screening test, designed to recognize anti HLA antibodies, which consists of beads coated with the 30 most common HLA class-I antigens to be analyzed on flow cytometry. HFE antisera at dilution 1:50-1:200 did not show detectable binding in the system (not shown). Mice produce small amounts of antisera, and this limits their use; this problem was overcome by inducing milliliter amounts of ascitic fluids in the immunized mice[27] with titers in Western blotting and immunoprecipitation similar to those of the corresponding antisera.

Immunohistochemistry of duodenum

In preliminary experiments we found that antisera from mice boosted with either HFE protein or with p3.1-HFE vector positively stained paraffin fixed duodenal tissue of HHC patients at proper dilutions (1:200-1:500), while they did not specifically stain other tissues, such as lymph nodes, liver, or brain (not

Table 1. Characteristics of the patients with hemochromatosis and control subjects.

Pts.	*Sex*	*Diagnosis*	*C282Y*	*Iron depleted at the time of biopsy*	*HH familial history*	*HI*	*IR (g)*	*HCV*	*HBV*	*Alcohol*	*HFE immunostain in duodenum*
ML	F	HH	+/+	Yes	yes	3.5	15	no	no	yes	granules
GE	F	HH	+/+	Yes	no	-	25	no	no	no	granules
PV	M	HH	+/+	No	no	5.6	30	no	no	yes	granules
AM	F	HH	+/+	No	yes	-	8	no	no	yes	granules
AD	M	HH	+/+	No	yes	-	25	no	-	no	granules
TG	M	HH	+/+	No	yes	3.6	13	no	no	no	granules
BA	M	HH	+/+	No	no	2.9	10	no	no	yes	granules
LU	M	HH	+/+	No	yes	5.5	15	no	no	no	stain
CC	F	HH	+/+	No	yes	2.3	5	no	no	no	stain
MA	M	HH	+/+	No	yes	4.6	nd	no	no	yes	stain
PV	M	HH	+/+	Yes	no	-	30	yes	no	yes	neg
CG	M	HH	+/+	No	no	3.7	20	no	no	no	neg
PB	M	HH	+/+	Yes	no	5.2	29	no	no	no	neg
FD	F	HH	+/-	Yes	no	-	7	no	no	no	neg
CC	M	HH	+/-	Yes	no	-	28	no	yes	yes	neg
ZA	M	HH	-/-	Yes	yes	3	8	no	no	yes	neg
SG	M	HH	-/-	Yes	no	-	6	yes	no	no	neg
VG	M	HH	-/-	No	no	2.3	8	no	no	yes	neg
VD	F	Pyrosis	-/-	-	-	-	-	no	no	no	neg
VC	F	Pyrosis	-/-	-	-	-	-	no	no	no	neg
FL	M	Pyrosis	-/-	-	-	-	-	no	no	no	neg
SE	M	Pyrosis	-	-	-	-	-	no	no	no	neg
LL	F	Pyrosis	-	-	-	-	-	no	no	no	neg

HI: hepatic iron index (hepatic iron concentration/age); IR: iron removed by phlebotomy (nd: non-depleted); HCV: hepatitis C virus; HBV: hepatitis B virus.

shown). A systematic immunohistochemical study was carried out on duodenal biopsies from 18 subjects with hemochromatosis, whose diagnosis had been based on the removal of > 5 g of iron to reach depletion, hepatic iron index > 2.3 (liver iron concentration /age) and a moderate to severe liver siderosis (Table 1). Of these biopsies, 10 were obtained at diagnosis when the subjects were iron overloaded (serum ferritin >1,000 mg/L) and 8 were obtained after iron depletion therapy had been completed and serum ferritin levels were below 50 µg/L. The subjects with hemochromatosis included 13 C282Y homozygotes, 2 C282Y homozygotes and 3 subjects with normal HFE alleles (Table 1). In addition, we analyzed biopsies from 5 subjects who underwent gastroendoscopy for pyrosis and had normal indices of iron metabolism and normal HFE alleles. In 7 of the 13 sections from C282Y homozygotes the antibodies produced a strong granular stain in the supranuclear region of all enterocytes in villi and crypts as shown in Figure 3A. In three other homozygotes a darker, non-granular stain was observed in the same regions, probably due to smaller granules (not shown), while in 3 homozygotes the granules were not detectable. In the tissues from the remaining five subjects with hemochromatosis, two C282Y heterozygotes and three with normal alleles, as well as in the five subjects without hemochromatosis, the antibodies gave a weak and diffuse stain, as shown in Figure 3B, similar to that obtained with pre-immune sera. The specificity of this diffuse stain was of difficult interpretation.

Discussion

Northern blotting analyses indicated that the duodenum contains high levels of HFE mRNA,[3] and indeed HFE has been immunohistochemically identified in duodenal crypt cells of normal subjects.[20,21] Here, HFE protein co-localizes with transferrin receptor, probably affecting its functionality in basolateral iron absorption.[21] Studies on transfected cells in which HFE protein is largely overexpressed indicate that HFE protein acts as an attenuator of TfR functionality[16,17,23] implying that the C282Y mutation of HFE increases transferrin-mediated cellular iron uptake. However, duodenal enterocytes in HHC appear to be abnormally iron deficient[30] suggesting that the decrease in HFE functionality due to C282Y mutation either decreases basolateral iron uptake in the proliferating crypt cells, or increases basolateral iron efflux in the mature enterocytes of the villi. An effect of the C282Y mutation on cellular compartmentalization was observed in transfectant studies,[9,13] however, there are no reports on its localization in the duodenum in HHC, despite the availability of various HFE antibodies. Our HFE antibodies differ from the ones already described by being elicited by the denatured recombinant protein and are likely to have higher affinity for non-native conformations of the protein, indicated by the sensitive recognition of denatured HFE in Western blotting. Antisera specificity was assessed by the lack of cross-reactivity with WS/32 antibody, and by the lack of recognition of HLA antigens in Western blotting and with the FlowPRA I system. The anti-

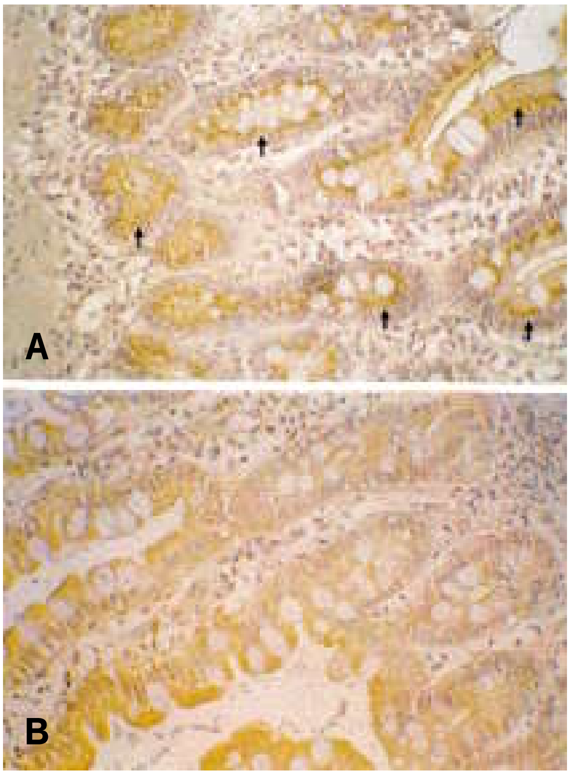

Figure 3. Immunostaining of duodenal slices. Duodenal slices were incubated with anti-HFE antiserum, the secondary antibody, then with avidin-biotin-immunoperoxidase and enzyme activity was revealed with 3,3-diaminobenzidine tetrahydrochloride. Panel A: duodenum of a subject homozygous for the C282Y mutation at 25x magnification, with granular staining in the crypts and villi indicated by the arrows. Panel B: duodenum of a subject with hemochromatosis with normal HFE alleles and without detectable granules at 25x magnification.

bodies have not provided evident specific signals in immunohistochemical tests of any of the tissues from normal subjects so far analyzed, including liver, lymph nodes and brain, which all express HLA antigens. Some diffuse stain was observed in the duodenum of non-HHC subjects, and in Kuppfer cells of the liver (not shown) but because of the low intensity and morphology we could not establish its specificity. Probably wild type HFE protein accumulates at low levels and likely diffuses in the tissues as in transfectant cell lines.[13] In addition, wild type HFE protein in immunohistochemistry may have a conformation with lower affinity for the antisera, and thus go undetected under the conditions used. The antisera produced a strong granular stain in the enterocytes of about 50% of C282Y homozygotes (7/13), and a weaker, albeit detectable stain, in another 27% homozygotes (3/13). This was obtained with antisera from mice boosted with HFE protein or HFE cDNA, with sera or ascitic fluids from the immunized animals, and was highly reproducible. Pre-incubation of the antisera with refolded HFE preparation significantly reduced the intensity of granular staining (not shown). Clearly, in immunohistochemistry our HFE antisera behaved differently from those used by Parkkila *et al.*,[20] which were elicited by synthetic peptides and gave positive signals in most tissues, including perinuclear staining of the epithelial cells of duodenal crypts.

This report, which appears to be the first on duodenal HFE protein in HHC, shows that in most C282Y homozygotes the protein accumulates in granular bodies which are uniformly distributed throughout enterocytes of crypts and villi (Figure 3A). This pattern is not evident in non-C282Y homozygous HFE, probably because staining is diffuse and below the detection limit of the system. A possible interpretation of the results is that the C282Y mutation, by abolishing the important structural disulfide bridge of the $\alpha3$ domain negatively affects the protein folding process and may induce protein aggregation, with the result of concentrating HFE and facilitating its detection. The mutated protein may assume non-native conformations more easily recognized by the antisera elicited by the denatured protein. We postulate that the aggregated HFE expressed in crypt cells may escape degradation and remain unchanged in the mature cells of the villi, thus explaining the even distribution of C282Y HFE granules in all enterocytes (Figure 3A). This is consistent with the observation that the C282Y mutant overexpressed in COS cells remains in high molecular weight aggregates.[9] Alternatively, the low affinity of C282Y mutant for TfR may lead to a redistribution of the protein from cell membranes to an intracellular pool. It remains to be noted that 3 out of the 13 C282Y homozygotes did not show evident granular or dark HFE staining, and that, at the time of analysis, two were iron depleted with serum ferritin levels < 50 μg/L, and one was untreated with serum ferritin >1,000 μg/L. Thus, HFE granular staining does not appear to be related to iron status or to any other obvious clinical finding. The explanations for this heterogeneous behavior of C282Y HFE mutant are presently unclear.

In conclusion, our data support the evidence that HFE in the duodenum is specifically expressed in enterocytes, and suggest that part of the loss of activity of HFE caused by the C282Y mutation may be attributed to protein accumulation/aggregation within the duodenal enterocytes. Further studies are needed to analyze whether granular deposition of HFE also occurs in other types of tissue in HHC.

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Contributions and Acknowledgments

LZ, BC and SL produced and anlyzed recombinant protein and tranfectants cells. ALF and SF followed the patients and collected the clinical data. MM performed the immunohistochemical studies. MS performed PCR experiments. AA collaborated in experimental planning and PA co-ordinated the work and wrote the paper. The order of authorship follows the relative contributions to the research. The authors are grateful to Dr. E. Puglisi for flow cytometry analyses.

Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

Manuscript processing

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Potential Implications for clinical practice

- An abnormal accumulation of HFE in duodenal enterocytes is often found in HFE-related hereditary hemochromatosis. This may affect duodenal iron absorption.

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