

Alternate Splicing Produces a Soluble Form of the Hereditary Hemochromatosis Protein Hfe

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ABSTRACT: HFE is a non-typical MHC class I-type protein that is mutated in hereditary hemochromatosis. The purpose of this study was to identify possible splice variants of HFE mRNA and investigate the regulation of these isoforms in duodenum and liver of patients with normal and altered iron stores. RT-PCR was performed using HFE specific primers and duodenal RNA obtained from patients with hemochromatosis, iron deficiency, secondary iron overload and normal controls. The reaction products were visualized by Southern blot and identified by DNA sequence analysis. Additional studies were performed on RNA isolated from liver and a range of human tissues. A truncated (soluble) form of HFE protein was identified that lacks the transmembrane domain and occurs as a result of alternative splicing. Soluble HFE was found predominantly in the duodenum, spleen, breast, skin and testicle. In hereditary hemochromatosis full length HFE was the predominant isoform present in the duodenum similar to iron deficiency. Alternate splicing produces soluble HFE that may have a unique function to regulate cellular iron transport. © 1999 Academic Press

INTRODUCTION

HFE is a major histocompatibility complex (MHC) class I-like protein that was found to have a C282Y mutation in 83% to 95% of typical hemochromatosis patients (1,2). A second less frequent mutation H63D was present in some of these patients. An HFE knockout mouse was produced and this animal model was found to develop iron overload in a similar tissue distribution to hereditary hemochromatosis, further supporting the role of HFE in the pathogenesis of this disease (3).

Significant insights into the cellular function of HFE have been provided by a number of studies. Transfection studies using human embryonic kidney cells (293 cells) and COS-7 cells showed that HFE bound β_2 microglobulin and was expressed on the cell surface, whereas C282Y HFE did not bind β_2 microglobulin and was localized to the endoplasmic reticulum and golgi (4,5). Later it was shown that HFE associated with transferrin receptor (TfR) on the cell surface of syncytiotrophoblasts and others found

using transfection studies that HFE decreased the binding affinity of transferrin (Tf) with TfR (6,7). In contrast, C282Y HFE did not associate with TfR or alter its binding affinity with Tf. These findings were supported by crystallographic studies that demonstrated HFE associated with TfR and Tf in a ternary complex (8). It was therefore proposed that the unregulated binding of Tf with TfR resulted in excess iron uptake by cells in C282Y associated hereditary hemochromatosis.

Despite these advances it remains unknown how HFE regulates intestinal iron absorption or indeed how C282Y HFE results in excess iron absorption. One immunohistochemical study localized HFE protein to duodenal crypt epithelial cells the site of dietary iron absorption, but no other reports investigating the expression or regulation of HFE in the duodenum have been published (9). Alternative splicing is a method of producing different isoforms of MHC class I proteins including soluble MHC

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protein which is important in the induction of immunological tolerance (10,11). The purpose of this study was to investigate the regulation of HFE mRNA expression in duodenum and liver of patients with normal and altered iron stores. In particular an examination of the intron/exon boundaries was performed to identify possible splice variants. This study was approved by the University of Western Ontario Review Board for Health Sciences Research involving human subjects.

METHODS

Biopsy specimens

Duodenal biopsies were obtained from four patients with normal iron stores, two patients with iron deficiency, three patients with secondary iron overload and eight patients with hereditary hemochromatosis (Table 1). Patients with hereditary hemochromatosis were C282Y homozygous (+/+) and control patients were wild type for C282Y (-/-). Liver biopsy specimens were obtained from four patients with hereditary hemochromatosis (three C282Y +/+, one C282Y +/-). Control liver biopsies were obtained from six C282Y -/- patients with minor abnormalities of liver function and four C282Y -/- patients with secondary iron overload. Specimens were also obtained of spleen, skin, breast, testis, fat and circulating leukocytes. Written informed consent was obtained from patients prior to the procedures being performed.

RNA isolation

Tissues were homogenized in TRIZOL™ reagent (Canadian Life Technologies Inc. Burlington, Ontario, Canada) and RNA was extracted with chloroform, precipitated with isopropyl alcohol and dissolved H₂O. Quantification and purity of RNA was determined by measuring absorbance at 260nm and 280nm.

First strand cDNA synthesis

First strand cDNA synthesis was performed using Superscript-II™ system (Canadian Life Technologies Inc. Burlington, Ontario, Canada) after DNase digestion of the RNA. Oligo(dT) primed (Canadian Life Technologies Inc.) reverse transcription was carried out using MMLV-reverse transcriptase at 42°C for 50 minutes in a total reaction volume of 20ul.

Control reactions were performed with Dnase digestion but without the addition of MMLV reverse transcriptase.

Polymerase Chain Reaction (PCR)

A HFE exon 4 sense primer (5'-AAGCAGCCAATGGATGCCAAGC-3') and a HFE exon 5 antisense primer (5'-TCCAATGAACAAGATGACGAC-3') were designed from the HFE gene sequence to produce a 243 bp fragment (1). These primers were used in 50µl reactions containing 1XPCR-buffer, 1.5mM MgCl₂, 250µM dNTP mix, 1µM of each primer, 2.5 U *Taq* polymerase and 2.5µl of the RT product. The reaction was denatured at 94°C for 4 minutes and then amplification was performed at 94°C for 45 sec, 55°C for 45 sec and 72°C for 60 sec for 40 cycles. Control PCR was performed using DNase digested RNA without the addition of MMLV reverse transcriptase. Simultaneously, β-actin was amplified in a separate reaction using the same amount of RT-product, 5'-TGGTGGGTATGGGTCAGAAGG-3' and 5'-ATCCTGTCAGCGATGCCTGGG-3' primers and similar cycling parameters to amplify an 840 bp product.

Automated DNA sequencing

DNA sequencing was performed using the ABI Prism™ BigDye™ Terminator Cycle Sequencing Ready Reaction Kit according to the manufacturer's instructions (Perkin Elmer Applied Biosystems, Mississauga, Ontario, Canada) by the J P Robarts Research Institute Sequencing Facility (London, Ontario, Canada). A sequence similarity search of the NCBI database (National Library of Medicine/National Institutes of Health, US) was performed on resulting sequences with the BLAST program.

Southern Hybridisation

The HFE RT-PCR product was visualised on an ethidium bromide stained gel and transferred to a nylon membrane. The RT-PCR product was confirmed to be HFE by hybridisation with an amplification specific biotinylated oligoprobe (5'-BioGGAAGAGCAGAGATATACGT-3'). An amplification product specific oligoprobe (5'-BiotCTGACCCTGAAGGTACCCCAT-3') was also

Table 1 Patient data, iron indices and HFE genotype

Duodenal biopsy group			
Hereditary Hemochromatosis	Serum Ferritin	C282Y	Clinical Diagnosis
1	3034	+/+	Untreated
2	2295	+/+	Untreated
3	291	+/+	Venesected
4	97	+/+	Venesected
5	893	+/+	Venesected
6	1140	+/+	Venesected
7	24	+/+	Venesected
8	61	+/+	Non-expressing
Control Patients			
1	101	-/-	Dyspepsia
2	117	-/-	Dyspepsia
3	89	-/-	Dyspepsia
4	165	-/-	Dyspepsia
5	18	-/-	Anemia
6	17	-/-	Anemia
8	3583	-/-	Alcoholic siderosis
9	925	-/-	Alcoholic siderosis
10	890	-/-	Thalassemia
Liver biopsy group			
Hereditary Hemochromatosis	Hepatic iron concentration	C282Y	Clinical Diagnosis
1	465	+/+	Untreated
2	69	+/+	Venesected
3	63	+/+	Venesected
4	137	+/-	Untreated
Control Patients			
1	58	-/-	Hepatitis B
2	75	-/-	Steatohepatitis
3	22	-/-	Drug hepatotoxicity
4	17	-/-	Hepatitis C
5	35	-/-	Drug hepatotoxicity
6	13	-/-	Steatohepatitis
7	298	-/-	Oral iron ingestion
8	82	-/-	Alcoholic siderosis
9	121	-/- (H63D +/-)	Alcoholic siderosis
10	275	-/-	Thalassemia

Serum ferritin normal 35-291µg/L, hepatic iron concentration normal 3.6-35.8 µmol/g, +/+ homozygous, +/- heterozygous, -/- homozygous wild type, C282Y and H63D mutations in the HFE gene.

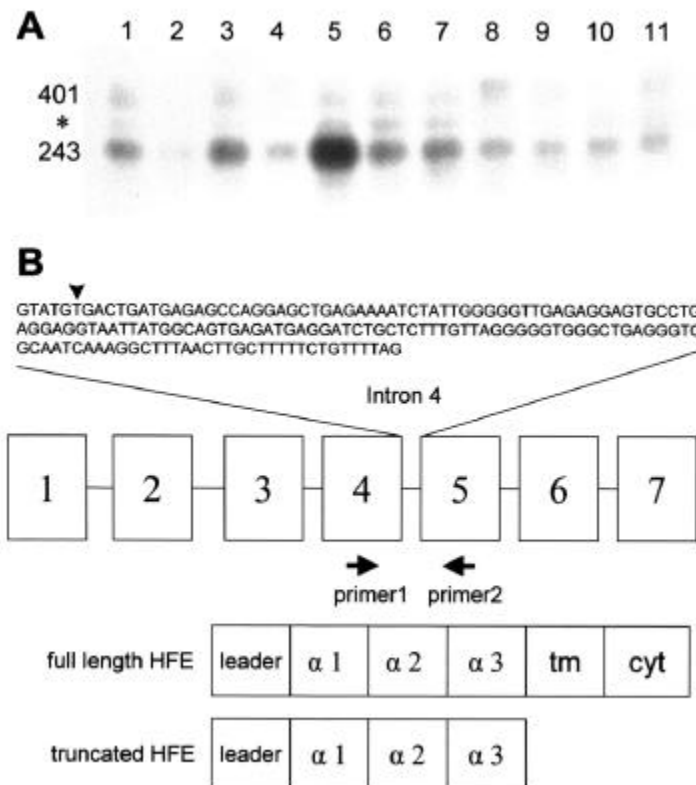


Figure 1. Alternatively spliced HFE in human tissues. (a) Total RNA was extracted from 1-esophagus, 2-antrum, 3-duodenum, 4-colon, 5-liver, 6-spleen, 7-breast, 8-skin, 9-fat, 10-circulating leukocytes, 11-testicle and used in RT-PCR using HFE primers (exon 4 and exon 5). Products were analyzed by Southern blot using a biotinylated HFE oligoprobe. 243-expected size of cDNA, 401-alternative spliced cDNA, *-hybrid cDNA consisting of one 243bp strand and one 401bp strand. (b) Schematic representation of the HFE gene and sequence of intron 4 that contains a TGA stop codon six nucleotides from the exon 4 boundary (). Full length HFE contains a leader peptide (exon one), α1 helix (exon two), α2 helix (exon three), α3 helix (exon four), tm-transmembrane domain (exon five) and cyt-cytoplasmic tail (exon six). The premature stop codon in the alternatively spliced form of HFE results in a truncated protein that lacks the transmembrane and cytoplasmic domains. Primer 1 and primer 2 show the position of the primers used in the PCR.

used for β -actin. Detection was carried out using the NBT/BCIP system (Sure Blot Blue Southern, Oncor, Gaithersburg, MD, USA). Quantification of the Southern blot analysis was performed by densitometry of the blot signal using a Hewlett-Packard 4C scanner and Mocha™ software (Jandel Scientific, California, USA). The ratio of the densitometric value of the 401 bp band compared to the 243 bp band was calculated for each reaction.

RESULTS

Southern blot analysis of the HFE duodenal RT-PCR product using exon 4 and exon 5 primers detected a 243bp band of the predicted size and an additional 401bp band (Figure 1a). No PCR product was amplified using DNase digested RNA without reverse transcriptase.

Sequence analysis of the 243bp fragment was identical with the published HFE cDNA whereas the sequence of the 401bp fragment confirmed the presence of a splice variant form of HFE mRNA (Figure 1b). The splice variant included intron 4 and this insert contained a premature stop codon six nucleotides from the exon 4/intron 4 boundary. This truncated (soluble) form of HFE lacked the transmembrane domain and cytoplasmic tail of the full length protein. Soluble HFE was present in most tissues and was relatively abundant compare with full length HFE in duodenum, spleen, skin and testicle (Figure 1a). Low or absent levels of expression relative to full length HFE were found in esophagus, antrum, colon and circulating leukocytes.

Densitometric analysis quantified soluble HFE mRNA expression relative to full length HFE mRNA levels in duodenal biopsies. The ratio of soluble HFE levels to full length HFE levels was more than 0.5 in four patients with normal iron stores and in two of these the ratio was 1.2 (Figure 2a). There was no difference in the relative abundance of soluble HFE in three patients with secondary iron overload compared to patients with normal iron stores. The least amount of soluble HFE mRNA relative to full length HFE was present in iron deficiency with ratios of 0.5 and 0.3. Interestingly in hereditary hemochromatosis soluble HFE mRNA was absent or present in low amounts relative to full length HFE (ratio less than 0.5) in all cases apart from one (Figure 2a). This patient had a ratio of soluble HFE to full length HFE of 0.66 and was iron loaded at the time of biopsy. Analysis of liver biopsies found that full length HFE was the predominant mRNA isoform

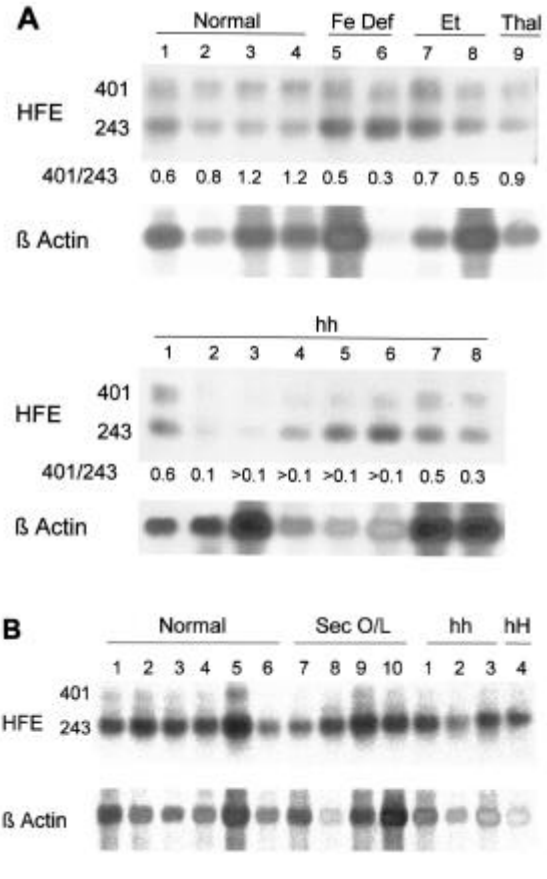


Figure 2. Regulation of alternative splicing of HFE. Southern blot analysis of RT-PCR using RNA from duodenal and liver biopsies. The HFE 401bp and 243bp products are shown as well as the 840bp β actin product. (a) Duodenal biopsies. Control patients 1 to 4 normal iron stores, patients 5 and 6 iron deficient, patient 7 and 8 (Et) alcohol abuse, patient 9 (Thal) thalassemia. C282Y +/+ hereditary hemochromatosis (hh) patients 1 to 8. Ratio of the densitometric value of the 401bp band to 243bp band shown. (b) Liver biopsies. Control patients 1 to 6 minimal liver dysfunction, patients 7 to 10 secondary iron overload. Hereditary hemochromatosis patients 1 to 4 (patient 4 C282Y +/-).

in all patients (Figure 2b). Meaningful densitometric analysis could not be performed due to the absent or negligible amounts of soluble HFE.

DISCUSSION

This study has demonstrated for the first time that HFE mRNA undergoes alternative splicing at the 5' splice site of intron 4 in human tissues. The 158 bp

insert contained a premature stop codon and the deduced amino acid sequence produced a truncated 294 amino acid isoform of HFE (mature protein of 272 residues) that terminated with Gly and Met residues after the α -three helix. This novel isoform of HFE is a soluble HFE protein that lacks the transmembrane domain and cytoplasmic tail of full length HFE. The inclusion of intron 4 resulted from the presence of an imperfect 5' consensus sequence at the exon 4-intron 4 boundary. Tissue specific regulation of alternate splicing was demonstrated with relatively abundant levels of soluble HFE in the duodenum, spleen, skin and testicle. In contrast the remainder of the gastrointestinal tract, breast, leukocytes and the liver had little if any soluble HFE detected compared with full length HFE. The relative lack of soluble HFE mRNA in liver may explain why previous investigators failed to identify this splice variant when HFE was cloned from a liver cDNA library (1). Moreover the inclusion of the 158 bp insert in soluble HFE mRNA would not be detected by northern blot assays as it would not significantly slow the rate of migration of the 4 kb transcript of full length HFE.

A number of proteins related to HFE utilize splice variants to regulate metabolic pathways. HLA G is a non-typical MHC class I protein with significant homology to HFE (1). This protein is expressed by placental trophoblasts and it is postulated that it prevents NK induced lysis of fetal cells (12). Soluble HLA G is produced in a similar manner to soluble HFE, with the inclusion of intron 4 that contains a premature stop codon. Metal transport proteins also use alternative splicing pathways to produce different protein isoforms. The Wilson disease protein mRNA undergoes alternative splicing and the two isoforms of this protein localized to different compartments of the cytoplasm. This suggested that these proteins are key regulators of copper metabolism and have distinct roles (13). Iron regulatory element (IRE) containing and non-IRE containing mRNA isoforms of the divalent metal transporter 1 (DMT1), previously called the divalent cation transporter 1, were found to be differentially expressed in the duodenum and other tissues (14). It was postulated that this mechanism allowed for the regulated uptake of iron by the enterocyte.

Others have independently examined the function of recombinant soluble HFE and found that this protein reduced the affinity of TfR for Tf and that it also bound tightly to soluble TfR and formed a ternary complex with Tf (7,8). It would therefore seem likely that the synthesis of soluble HFE in a

tissue dependent manner could have an effect on the regulation of cellular iron transport. In this study patients with C282Y+/+ hereditary hemochromatosis had small or negligible amounts of soluble HFE mRNA levels relative to full length HFE in duodenal mucosa and this was similar to two patients with iron deficiency that were studied. Soluble HFE may therefore be acting to associate with cell surface expressed TfR in remote tissues or circulating leukocytes and regulate the uptake of diferric Tf by these cells. The relative lack of this protein in hereditary hemochromatosis may help to account for the increased uptake of iron by tissues.

It was postulated that in hereditary hemochromatosis the lack of association of C282Y HFE and TfR in crypt enterocytes resulted in decreased uptake of diferric Tf (15,16). After migration these cells become absorptive epithelial cells of the villus and then regulate iron absorption (possibly by DMT1) as if the body were iron deficient. The physiological effects of soluble HFE need to be further examined to determine if this protein has a role in the regulation of dietary iron absorption and importantly if soluble HFE serum levels alter with changes in body iron stores and in hereditary hemochromatosis. A preliminary study has reported that soluble HFE was reduced in the serum of iron loaded rats compared to normal control rats (17). It will also be of interest to investigate whether the relative lack of soluble HFE mRNA in the duodenum of C282Y hereditary hemochromatosis patients is a result of this mutation or alternatively a consequence of the iron overload present in this disorder.

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