Identification of two novel mutations in the 5'untranslated region of H-ferritin using denaturing high performance liquid chromatography scanning

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Background and Objectives. Hereditary hyperferritinemia cataract syndrome is caused by mutations of the iron responsive elements (IREs) of L-ferritin mRNA. These alter the IRE structure and determine L-ferritin upregulation. IREs are located in 5'untranslated regions (5'UTR) of ferritin mRNAs. L-ferritin 5'UTR has been extensively studied and up to 21 different mutations have been identified. Only one mutation has been reported for H-ferritin 5'UTR; this mutation modified IRE structure and was apparently associated with high serum ferritin levels and iron overload.

Design and Methods. To identify other mutations in H ferritin 5'UTR we developed a fast DNA scanning method based on denaturing high performance liquid chromatography (HPLC). Five artificial DNA mutants were produced in order to validate the analytical conditions of the system for the identification of all mutations by single runs at 68°C. The system was used to screen 660 DNA samples from subjects with high serum ferritin levels.

Results. Two abnormal patterns were identified carrying the mutations C20G and G34T. Structural data and the analysis of ferritin levels in red blood cells suggest that these mutations do not affect the functionality of the IRE.

Interpretation and Conclusions. This large and first population analysis indicates that mutations in the H-ferritin 5'UTR are rare and do not seem to contribute to hyperferritinemia or iron overload.

Key words: iron metabolism, ferritin, hyperferritinemia, DNA variations, denaturing HPLC.

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Most of the iron-dependent regulation of gene expression in cells relies on the binding of the iron regulatory proteins (IRPs) to the iron responsive elements (IREs) located on 5' or 3 ' untranslated regions of mRNAs. Binding affinity is high when cellular iron is low and as a consequence represses the expression of genes with IREs in the 5'UTR or stabilizes the transcripts with IREs in the 3' UTR.[1] The opposite occurs when cellular iron is abundant. The system regulates the expression of more than 7 proteins, among which H-, L-ferritin and TfR1 play the most important roles in regulating iron homeostasis.[2] Up to 21 different mutations have been identified in L-ferritin 5'UTR; they include point/double substitutions and deletions of different lengths, some of which affect and disable IRE structures to different extents.[3] These mutations may cause a constitutive up-regulation of L-ferritin in serum and all tissues and are associated with a dominant genetic disorder named hereditary hyperferritinemia cataract syndrome.[4-6] These findings indicate that this DNA fragment is particularly prone to mutations.[7] H-ferritin gene has a structure very similar to that of L ferritin, including a rather long 5'UTR (208 and 205 nt for H and L-ferritin, respectively) and the stemloop structure of IRE and its position near the cap site of the transcript (the loop is at 48 and 39 nt for H-and L-ferritin, respectively). In fact the two ferritins are equally regulated by iron availability.[8]

One mutation in the H-ferritin IRE has been described in a Japanese family affected by dominantly transmitted iron overload and high serum ferritin levels.[9] The mutation consisted in the A49 T substitution within the loop of the IRE structure, which is known to be involved in IRP binding.[10]

Surprisingly, the mutation was found to increase binding affinity to IRP with a consequent down-regulation of H-ferritin expression in liver and increase in L-ferritin in serum.[9] A similar serum ferritin increase was found in mice with reduced H-ferritin expression caused by the inactivation of one allele: these animals did not, however, develop iron overload.[11] This finding raised the question of whether mutations in the 5'UTR of H-ferritin are frequent, and with which clinical manifestations they may be associated.

We had previously established denaturing high performance liquid chromatography (DHPLC) conditions to scan large numbers of DNA samples in order to identify heterozygous mutations in the 5'UTR of L-ferritin.[7] The system proved to be fast and reliable since it detect-

ed all the mutations. Here we report the development of a new DHPLC protocol specific for the 5'UTR of H-ferritin and its application to the screening of 660 DNA samples from subjects with high serum ferritin levels.

Design and Methods

Patients

DNA samples were obtained after informed consent from subjects with unexplained high ferritin levels, and from subjects who underwent HFE genotyping because of suspected hereditary hemochromatosis. HFE genotyping was performed as described elsewhere.[12] Genomic DNA was extracted from 10 mL of EDTA-anticoagulated blood using Qiagen-tip (Qiagen, Hilden, Germany).

Polymerase chain reaction conditions

Polymerase chain reaction (PCR) was performed in 50 µL containing 100 ng DNA, 200 µM each dNTP, 1.5 mM $MgCl_2$, 25 pmoles each primer and 1.5 U AmpliTaq Gold DNA Polymerase together with 1X PCR Buffer II (Applied Biosystems, Foster City, CA, USA). Cycling conditions were as follows: denaturation at 95°C for 10 min, 38 cycles at 95°C for 45 s, 57°C for 60 s, 72°C for 45 s and a final elongation step at 72°C for 5 min. Prior to DHPLC analysis, heteroduplexes were formed by denaturing the PCR product at 95°C for five minutes and cooling down to 56°C over one hour.

DHPLC mutational scanning

For DHPLC analysis the IREH-1 and IREH-2 primers reported in Table 1 were used, generating a PCR product of 246 bp which covers all the 5'UTR region of H-ferritin mRNA, before the start of transcription to codon 1. Mutation analysis was performed, according to a method described previously,[13] on a Transgenomic WAVE® System equipped with a preheated C18 reversed phase column based on non-porous poly(styrene/divinylbenzene) particles (DNASep™; Transgenomic). Five microliters of the PCR mixture were injected into the column and hetero- and homoduplexes were eluted with a linear gradient formed by mixing buffer A (0.1 mol/L triethylammine acetate pH 7.0) and buffer B (triethylammine acetate pH 7.0, containing 250 mL/L acetonitrile) at a constant flow rate of 0.9 mL/min. DNA was detected at 260 nm. The loading step was performed at 48% of B, while analytical gradient was 4 min long and buffer B was increased from 53% to 61%. The column was then cleaned with 100% buffer B for 30 s and equilibrated at starting conditions for 1 min.

The melting characteristics of the IRE-H DNA fragments were predicted using the Wavemaker™ software.

Table 1. Primers used for PCR amplification.

Code	*Use*	*Primer*
IREH-1	For. WT	5'-ACAAGCGACCCGCAGGGCCA-3'
IREH-2	Rev.WT	5'-GAGGTGGACGCGGTCGTCAT-3'
IREH-3*	For. Mut-1 **C→G**	5'-ACAAGCGACCCGCAGGG**G**CA-3'
IREH-4*	For. Mut-2 **C→A**	5'-ACAAGCGACCCGCAGGG**A**CA-3'
REH-5*	Rev. Mut-3 **G→C**	5'-GAGGTGGACGCGGTCGT**G**AT-3'
IREH-6*	Rev. Mut-4 **G→T**	5'-GAGGTGGACGCGGTCGT**A**AT-3'
IREH-7°	For. Mut-5	5'-TCAACAG**A**GCTTGGACGGAAC-3'
REH-8°	Rev. Mut-5	5'-GTTCCGTCCAAGC**T**CTGTTGA-3'
IREH-9	For. Sequencing	5'-CACCGGAAGGAGCGGGCTC-3'
IREH-10	Rev. Sequencing	5'-TAGAGCTCCAGGTTGATCTG-3'

**Designed to introduce the mutations indicated in bold at the termini of the amplicon. °Designed to introduce the mutation in bold the IRE-loop sequence underlined: CAGTG→CAGAG.*

Mutagenesis

In order to produce artificial positive controls, five different mutagenized amplicons were generated; four at the 5' and 3' termini of the DNA amplified fragment and a more central one. The 5' and 3' mutated controls (M1-M4) were produced by using mutagenized primers introducing either a conservative transversion (C→G) (IREH-3) and (IREH-5) or substitutions (C→A, IREH-4 and G→T, IREH-6) at the third base before the 3 ' end of each primer (Table 1). Conservative transversions are notoriously the most difficult sequence variations to visualize since they do not change the overall nucleotide composition and have little effect on the melting temperature of the fragment. The fifth control was constructed by introducing a conservative transversion T→A in the CAGUG sequence of the loop at position 51. This is similar to the previously reported A→T mutation in the second base of the IRE loop sequence.[9] The mutation was introduced by dividing each amplicon into two fragments: the 5' fragment was amplified with the original forward primer (IREH-1) and a mutagenizing reverse primer introducing a conservative transversion (IREH-8). In parallel, the 3' fragment was amplified with the original reverse primer (IREH-2) and a mutagenized forward primer (IREH-7) introducing the same nucleotide variation as above (Table 1). Cycling parameters and PCR conditions were the same as reported above. This led to the production of two partially overlapping fragments that were electrophoretically separated and

eluted in a final volume of 500 μL of distilled water each to eliminate non-incorporated primers. Two microliters of each eluted solution were mixed together and elongated for 15 cycles in a PCR reaction mixture containing all reagents but primers. An aliquot of 1 μL of the resulting full-length centrally mutagenized fragment was further PCR amplified for 20 cycles by adding the complete PCR mix including the two original forward (IREH-1) and reverse (IREH-2) wild-type primers. Direct sequencing confirmed the correct mutagenesis. After DHPLC analysis at 50°C under non-denaturing conditions in parallel with a wild-type amplicon, all the mutagenized and wild-type fragments were mixed in a 1:1 proportion based on the measurements of the areas corresponding to each elution peak to generate the artificial heterozygous control sample.

DNA sequencing

Direct sequencing was performed in both directions with the IREH-9 and IREH-10 primers reported in Table 1 using the DYEnamic ET Terminator Cycle Sequencing kit (Amersham Pharmacia Biotech) and a ABI Prism 3100 Genetic Analyzer (PE Biosystems, Foster City, CA, USA).

Ferritin determination in blood cells

Blood cell ferritin content was analyzed essentially as described by Piperno *et al.*[14] Cells were obtained from heparinized blood of a subject with a mutation in the H-ferritin 5'UTR and of two healthy subjects with normal iron status hematologic parameters. After lysis in a buffer containing 20 mM Tris HCl pH 8, 200 mM LiCl, 1 mM EDTA, 0.5% NP-40, the cells were frozen and thawed, and clarified by centrifugation. H- and L-ferritin content was evaluated by specific ELISA assays calibrated on the recombinant H and L homopolymers.[15] Protein concentration was determined by BCA assay (Pierce) calibrated on bovine serum albumin.

Results

Conditions of DHPLC analysis

The 5'UTR of H-ferritin has a high GC content (72%); Wavemaker™ software (Transgenomic) indicated an unusually high temperature of 68°C for DHPLC analysis (Figure1A). This suggested that the set-up of the analytical conditions to identify all mutations in the sequence might have been complicated. In fact, our previous attempts to develop DG-DGGE conditions for the same region were frustrating, since we did not reach an adequate band resolution even at the highest gel porosity and denaturing gradients (*not shown*). Therefore, we considered that it was essential to have controls to establish DHPLC conditions. In the absence of DNA samples carrying mutations in the region, we constructed them by PCR with modified oligonucleotides. These mutations were designed to mimic the most difficult situations: conservative G-C point substitutions at the termini (mutants 1 and 3) or in the middle of the sequence (mutant 5). This mutation is similar to the one reported by Kato *et al.*[9] Mixing equal amounts of mutated and wild type DNAs produced heteroduplexes with elution profiles clearly distinguishable from those of wild type homoduplex (Figure 1B). This indicated that the temperature of 68° C was adequate for the mutant identification. As a further control, we used direct sequencing to analyze the first set of 45 samples, all with a DHPLC pattern analogous to that of the wild type. The lack of false negative in the 90 alleles and the observation that the patterns of the 5 artificial (Figure 1B) and the two natural mutations (Figure 2A) are clearly different from those of the wild type, even though located in different parts of the amplicon (Figure 3), indicated that the system recognizes all mutations in the region.

This is a further demonstration of the great potential of DHPLC, which can be applied successfully to the analysis and population screening of difficult DNA sequences.

Population screening

We first analyzed a set of 550 DNA samples from patients who underwent HFE genotyping for the diagnosis of hemochromatosis. These samples had already been analyzed for mutations in the L-ferritin 5'UTR.[7,16] We found a single DHPLC abnormal pattern (Figure 2A). DNA sequencing revealed a G34→T mutation (Figure 2B). All the other samples showed normal patterns and were not further analyzed. The subject carrying the mutation had a marginally high serum ferritin value of 414 μg/L compatible with a marginally high transferrin saturation of 47%. Transaminases and liver markers were within the range of normality. Next, we analyzed another group of 67 subjects characterized by high serum ferritin levels associated with non-alcoholic fatty liver disease, a major cause of liver disease in Western countries, and one whose pathogenesis is related to insulin resistance. Hyperferritinemia, related to increased oxidative stress and mild iron overload, is a common clinical feature of the disorder. The mean age of these subjects was 51.4±11 years, 51 (76%) were males and 16 (24%) females. The mean serum ferritin was 630±306 μg/L, and transferrin saturation 39±15%. All the DHPLC patterns of these samples were normal, except one (Figure 2A), and DNA sequencing showed a C20→G mutation (Figure 2B). The patient was a 66-year old male with type 2 diabetes, who came to clinical observation because of hyperferritinemia (584 μg/L; normal value < 320) with normal transferrin

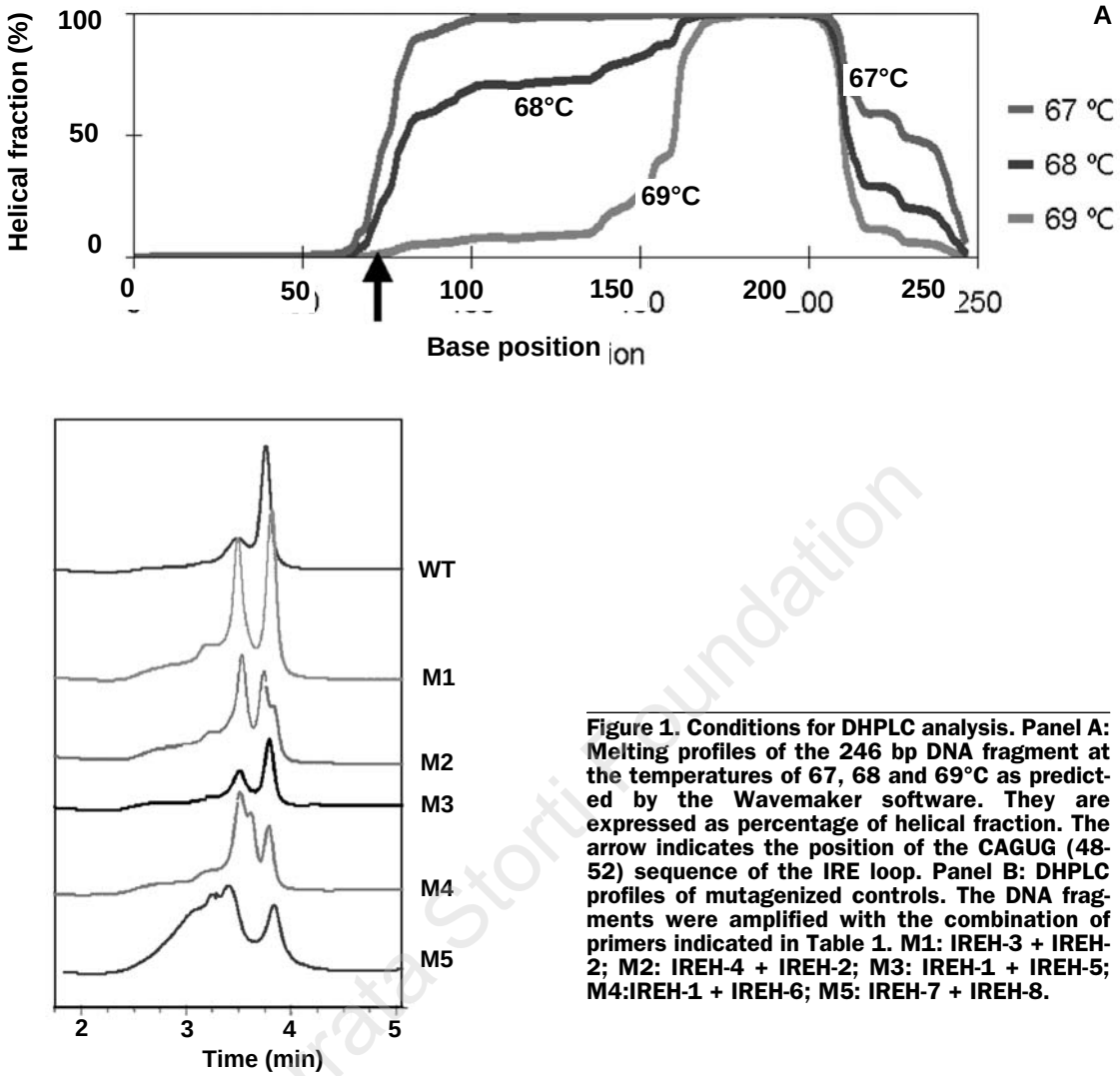

Figure 1. Conditions for DHPLC analysis. Panel A: Melting profiles of the 246 bp DNA fragment at the temperatures of 67, 68 and 69°C as predicted by the Wavemaker software. They are expressed as percentage of helical fraction. The arrow indicates the position of the CAGUG (48-52) sequence of the IRE loop. Panel B: DHPLC profiles of mutagenized controls. The DNA fragments were amplified with the combination of primers indicated in Table 1. M1: IREH-3 + IREH-2; M2: IREH-4 + IREH-2; M3: IREH-1 + IREH-5; M4:IREH-1 + IREH-6; M5: IREH-7 + IREH-8.

saturation (40%) and liver enzymes. The moderate degree of iron overload observed in this patient (he underwent phlebotomies and achieved depletion after removal of 2.5 g of iron) was probably related to heterozygosity for the HFE gene C282Y mutation and altered glycolipid metabolism; he also had a history of mild alcohol abuse in previous years. Family studies could not be carried out.

Functional analysis of the mutations

An indirect indication of the possible effect of the mutations can be obtained by aligning the 5'UTR sequence of the H-ferritin with that of the more characterized L-ferritin (Figure 3). This shows that H-ferritin C20G mutation is next to the L-ferritin C10T and C14G mutation, which did not have evident effects on ferritin expression;[16] in addition, it is located outside the stem (Figure 3A) and it can be reasonably concluded that this mutation has no, or only minor, effects on H-ferritin expression. More interesting is the G34T, which corresponds to position 25 of the L-Ferritin 5'UTR and is located in the lower stem of the structure (Figure 3). A neighboring mutation in the L-ferritin consists in the 22-27 deletion, which is likely to have long range effects on the IRE structure not comparable to point mutations.[17] A direct analysis of the IRP binding affinity of the mutant is difficult, because this is outside the typical IRE probe used to study IRP functionality.[18] We, therefore, used a more biological approach by analyzing ferritin levels in the most easily obtainable tissue, the blood cells.

ELISA assays showed that in the subject, H-ferritin concentration was 1.89 μg/g of total protein and L-ferritin concentration was 0.45 μg/g of total protein. In two control samples H-ferritin concentration was 1.01 and 1.40 and L-ferritin 0.44 and 0.34 μg/g total protein. Thus, the subject's H/L fer-

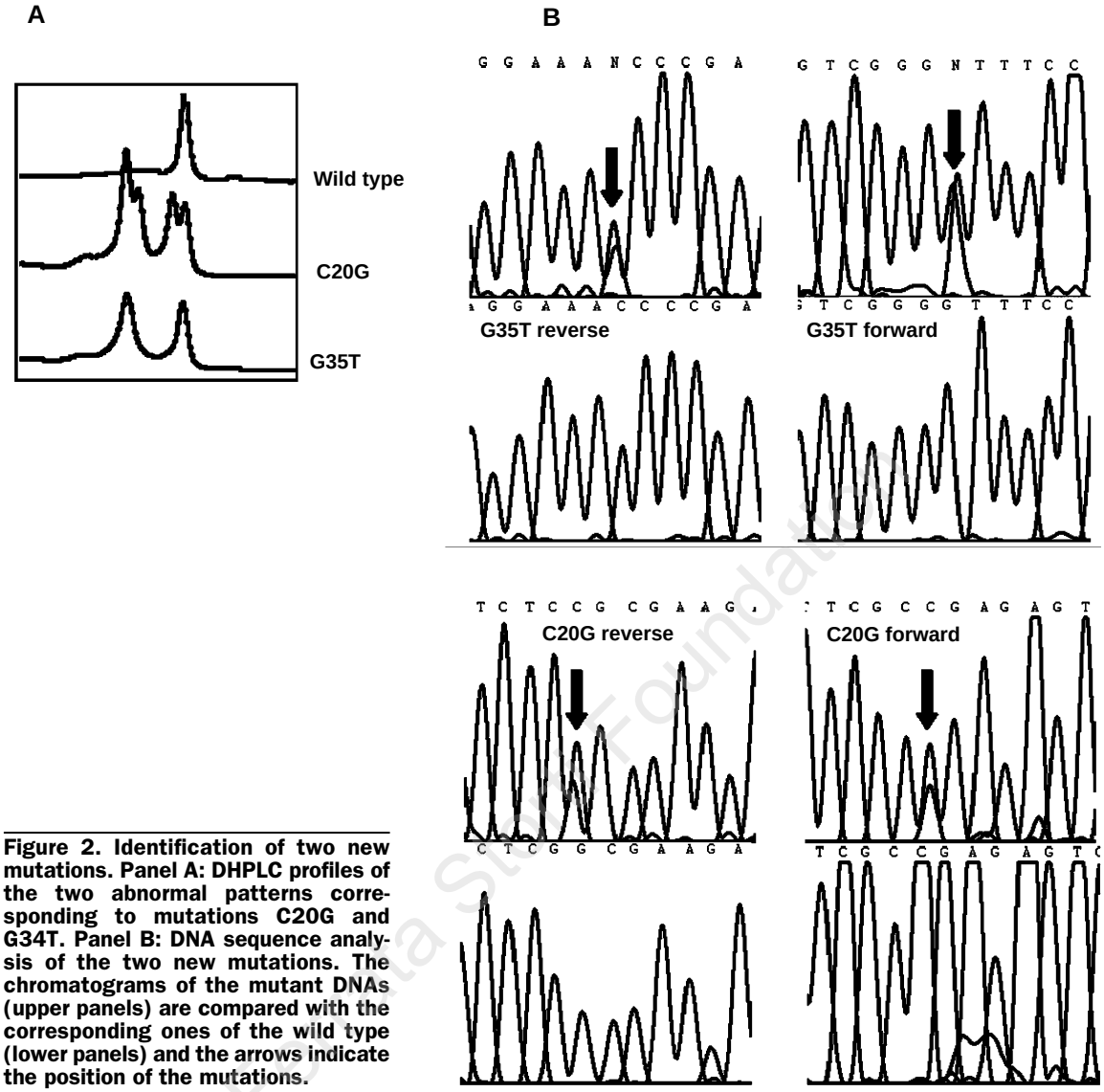

Figure 2. Identification of two new mutations. Panel A: DHPLC profiles of the two abnormal patterns corresponding to mutations C20G and G34T. Panel B: DNA sequence analysis of the two new mutations. The chromatograms of the mutant DNAs (upper panels) are compared with the corresponding ones of the wild type (lower panels) and the arrows indicate the position of the mutations.

ritin ratio was 4.2 and the ratios in the two controls were 4.1 and 2.3. These are all within the previously reported normal range of 0.6-4.1;[14] therefore, the mutation does not seem to have a major effect on H-ferritin level in blood cells. In conclusion, only 2 of the about 1320 alleles examined carried a mutation in the 5'UTR of H-ferritin. This frequency is much lower that that found in the same population for the corresponding region of L-ferritin (around 0.5%),[7] indicating that the 5'UTR of H-ferritin is much less prone to mutations than is that of the L-ferritin. In addition, we found that the two mutations are outside the the core of the IRE structure and they have no or only minor effects on H-ferritin expression.

Discussion

Our study population was characterized by high serum ferritin levels in order to verify whether mutations in the H-ferritin IRE might contribute to iron overload or hyperferritinemia, as indicated by Kato *et al.*[9] Our findings do not support this hypothesis. In addition, by similarity with L-ferritin it can be predicted that most mutations in the IRE structure[8] would result in a lower binding affinity to IRPs and in a constitutive up-regulation of H-ferritin. The phenotypic effects of such mutations are unknown and possibly do not involve high levels of serum ferritin or iron overload. Animal models with high H-ferritin expres-

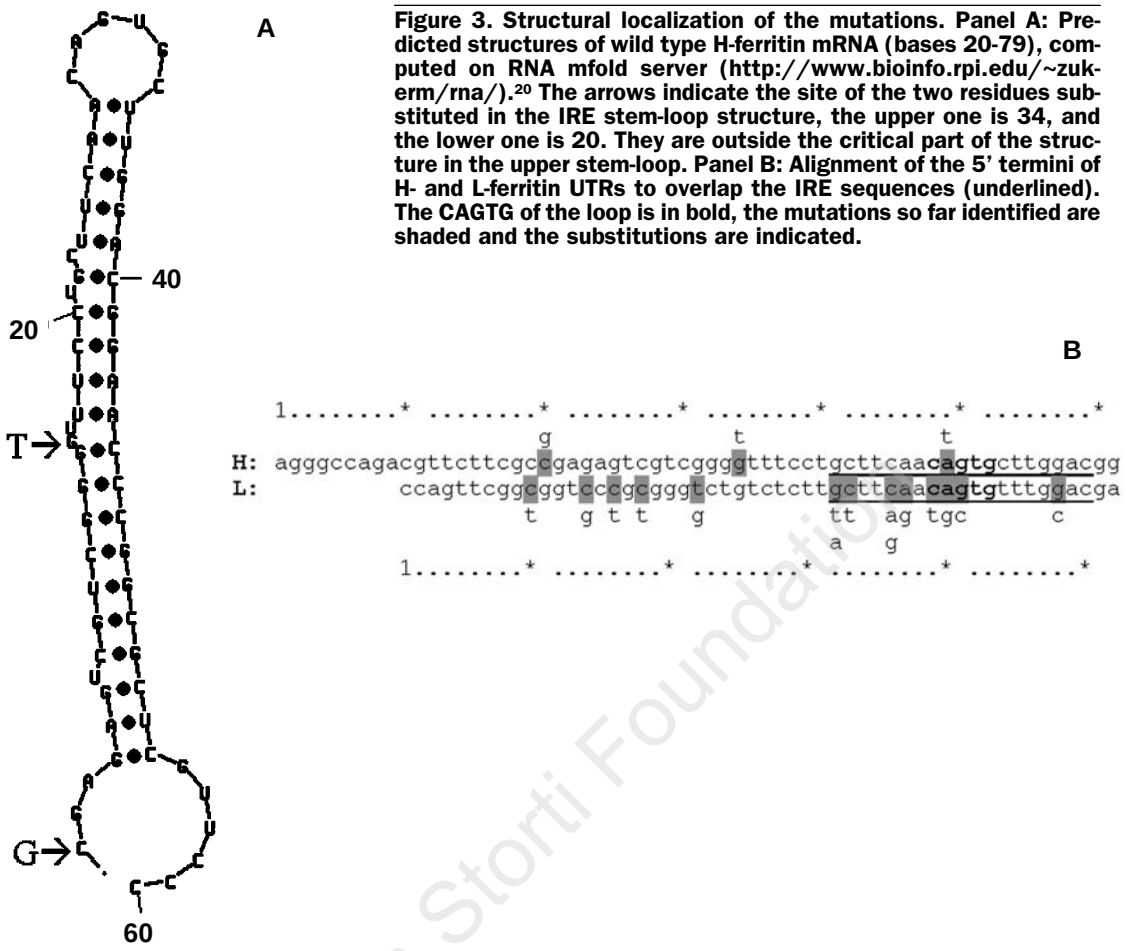

Figure 3. Structural localization of the mutations. Panel A: Predicted structures of wild type H-ferritin mRNA (bases 20-79), computed on RNA mfold server (http://www.bioinfo.rpi.edu/~zukerm/rna/).[20] The arrows indicate the site of the two residues substituted in the IRE stem-loop structure, the upper one is 34, and the lower one is 20. They are outside the critical part of the structure in the upper stem-loop. Panel B: Alignment of the 5' termini of H- and L-ferritin UTRs to overlap the IRE sequences (underlined). The CAGTG of the loop is in bold, the mutations so far identified are shaded and the substitutions are indicated.

sion have not been described, and cellular studies showed that upregulation of H-ferritin causes a reduction of iron availability, a reduction of proliferation rate, increased resistance to oxidative damage[15] and a reduction of hemoglobin synthesis.[19] Thus, it is possible that disabling mutations in the H-ferritin IRE are to be found in other population types, perhaps those with anemia or with an apparent iron deficiency. Alternatively, they might be so deleterious as to be incompatible with life. The availability of fast DHPLC scanning for mutations will help in the analysis of large population samples.

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Pre-publication Report & Outcomes of Peer Review

Contributions

All authors contributed to the design of the study. LC, BF, IF and RP contributed to DHPLC data collection and organization. AC and SL contributed mainly to biochemical analyses. SB, GR, SF and MF contributed to the collection of the DNA samples and clinical data. LC and PA acted as senior authors in designing the study, supervising the work and organizing the manuscript. All authors approved the final version of the manuscript. The authors are listed following decreasing individual contribution to the work. We are grateful to Prof. M. Cazzola for providing us samples.

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Disclosures

Conflict of interest: none.

Redundant publications: no substantial overlapping with previous papers.

Manuscript processing

This manuscript was peer-reviewed by two external referees and by Professor Mario Cazzola, Editor-in-Chief. The final decision to accept this paper for publication was taken jointly by Professor Cazzola and the Editors. Manuscript received August 1, 2003; accepted August 22, 2003.

In the following paragraphs, Professor Cazzola summarizes the peer-review process and its outcomes.

What is already known on this topic

In mammalian cells, cellular iron homeostasis is maintained by the co-ordinated regulation of transferrin receptor and ferritin synthesis that occurs at the translational level. This regulation is mediated by iron-responsive elements (IREs) that are found within the UTRs of mRNA, and by cytoplasmic mRNA-binding proteins, known as iron regulatory proteins (IRPs). Hereditary hyperferritinemia/cataract syndrome (HHCS) arises from various point mutations or deletions within a protein binding sequence in the 5'-UTR of the L-ferritin mRNA that results in increased efficiency of L-ferritin translation. So far, the only evidence of a translation genetic disorder of H ferritin synthesis has been provided by Kato and co-workers (*Am J Hum Genet 2001;69:191-7*). These authors reported the A49U mutation in the IRE of H-ferritin, suggesting that it was responsible for decreased H ferritin synthesis, elevated L ferritin production and tissue iron deposition.

What this study adds

This paper shows that mutations in the IRE motif of H-ferritin mRNA are very rare and anyhow very unlikely to be responsible for hereditary hyperferritinemia and/or iron overload. In addition, it can be predicted that most mutations in the IRE motif of H-ferritin mRNA (likely including also that described by Kato and co-workers) would result in a lower binding affinity to IRPs and in constitutive up-regulation of H ferritin.