Double-Gradient Denaturing Gradient Gel Electrophoresis Assay for Identification of L-Ferritin Iron-responsive Element Mutations Responsible for Hereditary Hyperferritinemia-Cataract Syndrome: Identification of the New Mutation C14G

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Background: Hereditary hyperferritinemia-cataract syndrome is an autosomic dominant disorder caused by heterogeneous mutations on the iron-responsive element (IRE) of ferritin L-chain mRNA. The mutations described to date were identified by direct sequencing of DNA from probands with hyperferritinemia often associated to bilateral cataracts. A direct genetic approach on a large population is useful to recognize polymorphisms in the DNA region and the prevalence of mutations associated with minor increases in serum ferritin and subclinical cataracts. We developed a rapid DNA scanning technique to detect mutations in a single electrophoretic analysis.

Methods: The double-gradient denaturing gradient gel electrophoresis (DG-DGGE) method consisted of PCR amplification of the target genomic DNA with GC-clamped oligonucleotides. The sequence encoded the 5' untranslated flanking region of ferritin L-chain mRNA, which includes an IRE stem-loop structure. The product was subjected to DG-DGGE (8.5–15% polyacrylamide and 50–95% denaturant) to separate the homo- and heteroduplexes.

Results: The method clearly identified all eight accessible mutations, including C-G transversions, which are the most difficult to detect. The method was applied to scan DNA samples from 50 healthy subjects and from 230 subjects with serum ferritin >400 μg/L. The new mutation G14C was identified.

Conclusions: The DG-DGGE method detects all the mutations in the L-ferritin IRE sequence, is rapid and economical, and can be applied to scan large populations. The first population study indicated that the mutations are rare and may involve regions of the IRE structure not yet characterized.

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Ferritin is an iron storage protein composed of two subunit types, H and L, which coassemble in different proportions in the 24mer protein, delimiting a large cavity where iron, up to 4000 atoms per molecule, is accommodated (1). The H chain has ferroxidase activity, whereas the L chain favors iron mineralization in the cavity. The two subunits accumulate preferentially intracellularly, but minor amounts are also found in body fluids and serum ferritin. Serum ferritin carries little or no iron and is composed almost exclusively of the L chain, part of which is glycosylated (2). The biological role of serum ferritin is obscure, but it is clinically useful because its concentrations are related to body iron status (3). The H and L chains are regulated differently at a transcriptional level but are regulated equally by iron at a translational level. The mRNAs for the H and L chains carry at the 5'-untranslated region terminus analogous stem-loop...
structures, named iron-responsive elements (IREs), which are designed to interact with specific iron-regulatory proteins (IRPs) (1). The binding prevents translation of the messengers and consequently down-regulates protein expression. In conditions of low cellular iron, IRP1 and IRP2 have high-affinity conformations for the IREs. In conditions of iron excess, the IRPs loose their affinity for IREs, and ferritin synthesis is derepressed. This leads to up-regulation of tissue and serum ferritin, the latter easily monitored by immunological methods.

Increased serum ferritin concentrations often are indicators of excess iron stores; however, several hyperferritinemic conditions are not related to iron overload, and they include disorders such as inflammations, neoplasia (3), and the recently described metabolic dysfunction-associated liver iron overload syndrome (4), conditions that are associated to iron decompartmentalization and characterized by low or normal transferrin saturation. A distinct and more specific cause of hyperferritinemia is associated with deregulation of ferritin synthesis, as it occurs in hereditary hyperferritinemia-cataract syndrome (HHCS; OMIM 600886). This is a genetic, autosomal dominant disorder caused by heterogeneous mutations in the IRE of L-ferritin mRNA. The mutations abolish or reduce the affinity with the IRPs, and this determines a constitutive up-regulation of ferritin L-chain in serum and all tissues (5, 6). The 10- to 20-fold higher accumulation of L-ferritin in serum and tissues has no evident effects on body iron status, but it often is associated with early bilateral cataract onset, likely via aggregation of the protein in the lens (7, 8). Thirteen different mutations have been described to date in 15–20 families (Table 1), including one sporadic case (9). The mutations span over a 30-nucleotide stretch that is part of the IRE structure, with some affecting the loop that interacts directly with the IRPs, whereas other mutations affect the stems or the bulge of the structure and modify the conformation of the IRE, leading to lower affinity binding (10). The in vitro effects of the mutations on IRE structures were found to be correlated with clinical symptoms: subjects with mutations that affect the upper part of the structure, which is involved in IRP binding, have ferritin in the 1000–1500 µg/L range and early-onset cataracts, whereas the mutations that affect the distal parts of the stems are associated with slightly increased serum ferritin (~500 µg/L) and subclinical cataracts (10). This finding suggests that mutations may occur that have clinically undetectable effects on serum ferritin and cataract formation. These mutations cannot be easily identified with the method used at present, which consists of direct sequencing of PCR-amplified DNA from patients with abnormal and unexplained serum ferritin concentrations, either associated or not associated with early onset of cataracts.

For a more complete analysis of the prevalence of mutations in this locus and the identification of possible polymorphisms, a faster and less expensive method than direct DNA sequencing is needed. A suitable approach consists of a fast mutational scanning technique that identifies all samples with mutations to be verified by direct sequencing.

The available scanning techniques consist of single-strand conformation polymorphism (11), denaturing gradient gel electrophoresis (DGGE) (12, 13), chemical cleavage of mismatch (14), RNase cleavage (15), and heteroduplex analysis (16), which identify only a portion of the mutations; among these methods, DGGE is the most effective with a detection rate close to 100% (17). The technique has been further improved by superimposing a porous gradient on the denaturing gradient [double-gradient DGGE (DG-DGGE)], which minimizes band broadening, even in prolonged runs, and permits more accurate band separation (18, 19). The DNA stretch of interest for mutations in HHCS is approximately the optimal size for this type of technology, which allows mutational scanning in a single electrophoretic analysis.

We describe the development of the assay, showing that it is able to identify without ambiguity all of the eight mutations tested. The assay was used to analyze DNA from 50 healthy subjects and 230 subjects with abnormalities in markers of iron metabolism. A new mutation was identified.

### Materials and Methods

**Patients**

Blood samples were obtained after informed consent from 50 healthy subjects and from 230 subjects who underwent HFE genotyping for diagnosis of hereditary hemochromatosis.
tosis. All 230 subjects had serum ferritin concentrations >400 μg/L (range, 420-5500 μg/L); transferrin saturation was <30% in only 11 subjects. HFE genotyping, performed as described by Carella et al. (20), identified 19 subjects homozygous for the C282Y mutation, 9 for the H63D mutations, 7 compound heterozygous, 91 heterozygous for either mutations, and 104 without either mutation. In addition, 50 subjects were positive for hepatitis C antigen and 18 for hepatitis B surface antigen.

**Primer Design and PCR Conditions**

The optimal PCR set of primers for GC-clamped DG-DGGE analysis of the IRE sequence was selected using the MELL87 program, kindly provided by Dr. L.S. Lerman (MIT, Cambridge, MA) (12). Primers were designed to allow the entire L-ferritin IRE sequence to span over a single melting domain with T_m of 78 °C. The PCR reaction mixture was as follows: 1 μg of DNA, 200 mM dNTPs, 10 mM Tris-HCl (pH 8.8), 50 mM KCl; 1.5 mM MgCl_2, 1 mL/L Triton X-100, 2.0 U of thermostable DNA polymerase (Dynazyme; Finnzymes OY, Celbio), and 30 pmol of each primer in a total volume of 100 μL. PCR cycles were as follows: 1 cycle at 94 °C for 5 min, followed by 30 cycles of 45 s at 94 °C, 45 s at 56 °C, and 45 s at 72 °C. The PCR product was 315 bp in length. Heteroduplexes were generated at the end of each PCR session by 5 min of denaturation at 94 °C and 1 h incubation at 56 °C.

**Optimization of DG-DGGE Conditions**

The amplified DNA samples (15 μL) were subjected to electrophoresis in a 8.5–15% polyacrylamide linear porosity gradient gel (acylamide:bisacrylamide, 39:1 ratio), in Tris-EDTA-acetate buffer (40 mmol/L Tris, 20 mmol/L NaOH, 1 mmol/L EDTA, titrated to pH 7.6 with acetic acid). The denaturing gradient was a 50–95% linear gradient of a mixture of urea and formamide (100% denaturant: 7 mol/L urea and 400 mL/L formamide). The gel slabs were 0.75 mm thick, 150 mm wide, and 16 cm long. At the end of an 18-h run at 75 V, the gels were stained in ethidium bromide.

**Fluorescent Sequence Analysis**

Sequencing was carried out in both directions using fluorescent dyeoxy chain terminator cycle sequencing on an ABI PRISM 377 DNA sequencer (Perkin-Elmer). Specific primers for sequencing were designed external to those used for DG-DGGE as follows: forward primer, 5’-TCCT-TGCCACCGCAGTTGG-3’; reverse primer, 5’-CTGAC-GAATCTGGGAGCTCA-3’.

**Results**

**Development of the DG-DGGE Assay**

The IRE of L-ferritin mRNA consists of a stem-loop structure located near the 5’ terminus of the untranslated region of the transcripts spanning more than ~50 residues; all the mutations identified to date are between positions 18 and 57 (Table 1). We considered that the analysis of a 200-residue stretch from the start of translation was sufficient to recognize all mutations that may affect IRE functionality. The region is not interrupted by introns and can be analyzed by PCR amplification of genomic DNA. To set up the DG-DGGE assay, we first analyzed the melting profile of the sequence using the MELL87 program (12) to identify a region covering the whole IRE sequence that had a single melting domain with a T_m of 78 °C. GC-clamped oligonucleotides to amplify this region were synthesized, and PCR conditions and DG-DGGE runs were optimized with the aim to identify all mutations in a single electrophoretic analysis. Because of the high G and C content, which accounts for the high temperature melting profile of the IRE sequence, we used a narrow highly denaturing gradient (50–95%) to maximize homo- and heteroduplex separation.

**Analysis of Known IRE Mutations**

To evaluate the scanning efficiency of the method, we analyzed DNA samples from eight HHCS subjects carrying mutations that had already been characterized (Table 1). All of them produced electrophoretic patterns clearly different from the single sharp band of the controls (Fig. 1A). The mutations were characterized by three extra bands, one corresponding to the mutated homoduplex and two weaker bands corresponding to heteroduplexes with lower melting points and slower mobility. The method was also able to reveal G-C transversions (Fig. 1A, lane 9), which are notoriously difficult to identify.

All of the patterns could be interpreted on the basis of the known mutations. For example, the lack of heteroduplexes in lane 8 (Fig. 1A) was expected for the large deletion delC10-A38, as were the remarkably low melting points of the heteroduplexes in the double substitution C18T + T22G (Fig. 1A, lane 6). The A40G mutation is expected to stabilize double-stranded DNA, and in fact its mutated homoduplex melting point was higher than that of the wild type (Fig. 1A, lane 5). The only sample that produced a pattern that could not be easily interpreted was the one of lane 3 of Fig. 1A because the G41C mutation was predicted to produce a homoduplex with a melting point very close to the wild type, like that of lane 9, and not the one we observed. Direct sequencing revealed that in fact the DNA contained an extra mutation in the adjacent residue, A40G (Fig. 2), which was not detected before, and explains the lower stability of the homoduplex.

**Population Scanning**

To validate the techniques and to evaluate the possible existence of polymorphisms, we first scanned a control group of 50 DNA samples from healthy subjects with iron metabolism indices within reference values. No abnormal electrophoretic patterns were observed, indicating a prevalence of polymorphisms <1%.

We then analyzed 230 DNA samples from subjects with serum ferritin concentrations ranging from 420 to...
5500 μg/L who underwent HFE genotyping for diagnosis of hereditary hemochromatosis. The high serum ferritin concentrations were attributed to iron overload or to liver disease, and only 12 subjects had transferrin saturation <30%. All of them showed single-band normal electrophoretic patterns in the DG-DGGE assay, except one (Fig. 1B, lane 1), which had an extra, close-running heteroduplex. DNA sequencing revealed the mutation C14G (Fig. 1C), the location of which is shown in Fig. 2. The subject had high serum ferritin (1200 μg/L) and high transferrin saturation (96%), whereas Perl’s staining of the liver biopsy showed no evident iron accumulation. The abnormal indices of iron status were attributed to the presence of hepatitis, indicated by the positivity to hepatitis B virus. This patient showed no signs of cataracts. Family studies could not be performed.

**Discussion**

The DG-DGGE method we describe allows rapid mutational scanning of the L-ferritin IRE sequence in a single electrophoretic analysis. We had access to DNA samples with eight previously identified mutations, and all of them were unambiguously recognized by the assay (Fig. 1A). Other mutations that we had no access to were C36A (21), deletion A38-C39, and deletion U42-G57 (22), which can be predicted to produce easily separable homoduplexes with lower melting points. More important is the evidence that the method clearly identified C-G conservation.
Conservative transversions, which do not alter overall base composition and have a minor effect on DNA $T_m$ (Fig. 1A, lane 9). These transversions are known to be the most difficult mutations to detect in scanning techniques because the two homoduplexes often are not separated and recognition is based on identification of the weak heteroduplex bands, which can be ambiguous. The finding that the method is able to recognize all tested mutations in the region made us confident that it can distinguish all possible mutations in the DNA stretch of the L-ferritin DNA that encodes for the complete IRE structure. An additional advantage of the method includes the deductions that can be made based on the electrophoresis patterns, which complement the results of DNA sequencing. This was useful at least in one case and allowed us to correct an error by demonstrating that the described point mutation G41C (23) is really a double mutation (G41C + A40G; Fig. 1A, lane 3). Reanalysis of DNA from the family members confirmed that all of the affected subjects carry the double mutation (not shown).

The DG-DGGE technique is designed for the scanning of large samples, and we first applied it to the analysis of 100 alleles from 50 healthy controls. No abnormal patterns were observed, and with the assumption that the method has 100% sensitivity, a prevalence of mutations or polymorphism <1% was deduced. The analysis was then extended to 230 samples from subjects with some abnormalities in iron metabolism who underwent HFE genotyping for diagnosis of hemochromatosis. In principle, this is not a population at risk for HHCS because the abnormal serum ferritin concentrations are attributed to iron overload. However, we decided to analyze the group because serum ferritin is an important marker for the diagnosis of hemochromatosis, and it has previously been reported that subjects with HHCS had been diagnosed with hemochromatosis (23). The DG-DGGE assay identified one abnormal electrophoretic pattern (Fig. 1B), and direct DNA sequencing revealed the new mutation, C14G (Fig. 1C).

The mutation C14G abolishes a base pairing in the lower part of the stem, and it is expected to affect the conformation of the IRE structure. In fact, structural modeling predicts a folding stability of $-25$ kcal/mol, compared with $-29.7$ kcal/mol for the wild type (24). A direct experimental in vitro study of the effects of the mutation on IRP binding would be complex because this region of the IRE structure has not been explored and electrophoretic mobility shift assays for IRE activity use RNA fragments from residue 26 to residue 55, which excludes this portion (25). The subject that carries the mutation had high serum ferritin, which was partially explained by iron overload, as indicated by the high percentage of transferrin saturation. Ophthalmologic analysis excluded cataracts, and genetic analysis did not identify HFE mutations. We could not perform family studies; thus, we do not have direct evidence that the mutation has phenotypic expression. The mutation structurally closest to this one (C18U + U22G) was shown to have mild clinical effects, with serum ferritin concentrations $\leq 500 \mu g/L$ and subclinical cataracts (5); by analogy the C14G mutation may have a minor effect on serum ferritin or be the first polymorphism of the region identified to date.
In conclusion, we demonstrate that the DG-DGGE technique we developed is adequate for the scanning of large populations for L-ferritin IRE mutations and that mutations and polymorphisms in the region are rare. The method allows more accurate definition of the prevalence of the mutations in populations with juvenile, bilateral, and congenital cataracts and in populations with hyperferritinemia. We speculate that it is particularly suitable for the study of translational disorders in which the DNA segment exposed to mutation is sufficiently short to be scanned in a single electrophoretic analysis. HHCS is the major example of these types of diseases, but IRE structures are present on the mRNA for H-ferritin, mitochondrial aconitase, ALAS1, IREG1, DMT1, and also transferrin receptor-I. Their mutations have not been explored, although they may have serious pathological consequences (26). In addition, hereditary thrombocytopenia is a translational disorder characterized by mutation in the regulatory 5′ untranslated region of the TPO gene, a region that can be efficiently scanned with DG-DGGE (26).

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