Improved Molecular Diagnosis of Hereditary Hemochromatosis Using a DNA Enzyme Immunoassay, Maria Raffaella Biasini,1 Tosca Bertin,2 Giuseppe Sardeo,3 Paolo Farris,4 Enzo Venza, and Domenico Infantolino5 (1 U.O. Istituzione ed Anatomia Patologica, Ospedale Civile, 31033 Castelfranco Veneto, Italy; 2 Medicina III, Ospedale “S Bortolo”, 36100 Vicenza, Italy; 3 Medicina I, Ospedale Civile, 31044 Montebelluna, Italy; 4 U.O. Malattie Infettive, Ospedale “S Bortolo”, 36100 Vicenza, Italy; 5 address correspondence to this author at: Modulo di Diagnostica Molecolare, U.O. Anatomia Patologica, Ospedale Civile, 31033 Castelfranco Veneto, Italy; fax 39-423-732280, e-mail infantolino@ulssasolo.ven.it)

Hereditary hemochromatosis (HH) is an autosomal recessive disorder of iron metabolism characterized by excessive absorption of dietary iron from the small intestine, leading to gradual accumulation in several organ systems (1).

In the Caucasian population, HH affects ~3–8 in 1000 individuals, with an estimated prevalence of heterozygous carriers of 1 in 10 (2). The molecular basis for HH was completely unknown until the identification by Feder et al. in 1996 of a gene on chromosome 6p, designated HLA-A. The gene, subsequently renamed HFE by the Nomenclature Committee of the Genome Database, is a MHC class Ib gene.

A single missense mutation, a G-to-A transition at nucleotide 845 that produces a cysteine-to-tyrosine substitution at position 282 (C282Y) in the HFE protein, has been observed in the majority of HH patients, with frequencies ranging from 64% to 100% in different geographic areas. The role of a second mutation, which produces a histidine-to-aspartic acid substitution at position 63 (H63D), remains controversial, although it is clearly associated with HH.

The strategies developed to screen HH chromosomes for the C282Y (845A) mutation include allele-specific oligonucleotide hybridization (4), restriction enzyme analysis (5), and oligonucleotide ligation assays (3). However, the use of all of these techniques is still confined to research laboratories because they are relatively expensive, time-consuming, and complicated for routine screening. In this report, we describe a PCR-based method that uses a DNA enzyme immunoassay (DEIA) for the specific detection of C282Y mutations.

After receiving informed consent, we analyzed 75 subjects. Of these, 46 were unrelated cases with a suspicion of hemochromatosis, whereas the remaining 29 subjects were members of five families with HH, comprising five probands and their relatives. Patients with HH had been diagnosed previously according to clinical criteria (signs and symptoms, increased transferrin saturation and/or serum ferritin, and presence of parenchymal iron overload on liver biopsy specimen).

After genomic DNA was extracted from peripheral blood leukocytes, 200 ng of template was amplified by PCR using 50 pmol of forward (5′-TGG CAA GGG TAA ACA GAT CC-3′) and reverse (5′-TCT AGG CAC TCC TCT CAA CC-3′) primers according to Feder et al. (3) in 100 μL of reaction mixture containing 2.5 U of Taq DNA Polymerase (Life Technologies), 1× PCR buffer (20 mmol/L Tris-HCl, pH 8.4, 50 mmol/L KCl), 200 μmol/L deoxynucleotides (Amersham Pharmacia Biotech), and 3 mmol/L MgCl2. Amplification was as follows: initial denaturation of 94 °C for 2 min, followed by 30 cycles of 94 °C for 40 s, 55 °C for 40 s, and 72 °C for 40 s (additional 5 min in the last cycle). All samples were tested for the HFE genotype by restriction enzyme analysis and further analyzed by DEIA as follows. Briefly, streptavidin-coated microtiter plates (ETI-IEMA; DiaSorin) were incubated overnight at 4 °C with 40 ng/well of biotinylated oligonucleotide probes in 100 μL of Tris-EDTA (10 mmol/L Tris, pH 8.0, 1 mmol/L EDTA). Each amplified specimen was added to a well coated with the 845G capture probe (5′-GAT ATA CGT GCC AGG TGG-3′), specific for the wild-type allele, and a second well coated with the 845A oligonucleotide (5′-GAT ATA CGT ACC AGG TGG-3′), specific for the mutant allele. Each test routinely included control samples for C282Y homozygotes, heterozygotes, and wild types. Two additional blank wells containing only chromogen/substrate were also included to check for cross-contamination. The solid phase was washed five times with 300 μL of washing solution containing 6.7 mmol/L phosphate buffer, pH 6.4, 0.13 mol/L sodium chloride, 0.04 g/L Cialit, and 1 mL/L Tween 20. The crude thermally denatured PCR products (15 μL) were added to the coated wells and incubated at 50 °C for 1 h in 100 μL of hybridization buffer (1× standard saline citrate, 2× Denhardt’s solution, 10 mmol/L Tris-HCl, pH 7.5, and 1 mmol/L EDTA). After five washes with washing solution, hybrids between the allele-specific probes and the HH gene sequences were detected by the addition of 100 μL of a 1:100 dilution of a standard preparation of anti-DNA monoclonal antibody (MAb 27-14-D9; DiaSorin) in phosphate-buffered saline containing 100 mL/L fetal calf serum; this monoclonal antibody recognizes only double-stranded DNA. At the end of a 30-min incubation at room temperature, the wells were washed five times, and 100 μL of an enzyme tracer [horseradish peroxidase conjugated to protein A (DiaSorin) diluted 1:20 000 in phosphate-buffered saline containing 100 mL/L fetal calf serum] was added to each well to reveal the bound antibody; the plates were then incubated another 30 min at room temperature. After the wells were washed five times to remove the samples, positive reactions were detected by a colorimetric reaction, which involved addition of 100 μL of ETI-IEMA chromogen/substrate mixture (27 g/L tetramethylbenzidine, 0.1 mmol/L hydrogen peroxide) and incubation in the dark for 30 min at room temperature. The reaction was stopped with 200 μL of 0.5 mol/L sulfuric acid, and the genotype of each amplified sample was detected by measuring the absorbance at 450 nm with a spectrophotometer.

Patients homozygous for the C282Y mutation were unambiguously identified by development of color only in the wells containing the 845A-specific probe. Samples from homozygous wild-type individuals gave a positive reaction only with the 845G-specific probe, whereas het-
The gold standard for the evaluation of the glomerular filtration rate (GFR) is inulin clearance (CIn), but its widespread use is prevented by several technical difficulties (1). The most commonly used marker for GFR is serum creatinine. However, serum creatinine concentrations should be interpreted with caution as a filtration marker in liver cirrhosis because they do not adequately reflect renal dysfunction. Increased tubular secretion and muscle wasting account for the disparity between creatinine concentrations and GFR in cirrhotic patients (2–4). Thus, GFR has been demonstrated repeatedly to be overestimated by serum creatinine (1–5).

Cystatin C, a cationic 13-kDa protein that is produced by nucleated cells and catabolized by the renal tubular cells after passing the glomerular filter, has recently been reported as a reliable endogenous marker of GFR in healthy adults and children as well as in patients with nephrologic, urologic, and rheumatologic disorders (6–9). For patients with liver cirrhosis, however, no data are available. Therefore, we studied cystatin C in comparison to creatinine for the assessment of GFR in these patients. We also determined the precision (10) of each analyte to predict GFR in cirrhotic patients.

Forty-four patients with liver cirrhosis who were clas-