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An HFE Intronic Variant Promotes Misdiagnosis of Hereditary Hemochromatosis

To the Editor:

Hereditary hemochromatosis (HH; MIM 235200), an autosomal recessive disorder of iron metabolism, can result in numerous clinical complications and is estimated to affect ~1/300 individuals of northern European origin (Merryweather-Clarke et al. 1997). Two mutations—C282Y and H63D—that contribute to HH have been identified (Feder et al. 1996), and screening for the C282Y mutation, in particular, is routinely done to identify carriers and affected individuals. Biochemical markers indicate a relatively clear distinction between these two groups, with minimal clinical consequences for heterozygotes (Bulaj et al. 1996). We initiated screening for the C282Y mutation, using the primer sequences provided by Feder et al. (1996) and subsequent restriction digestion of PCR products (Jazwinska et al. 1996). Re-

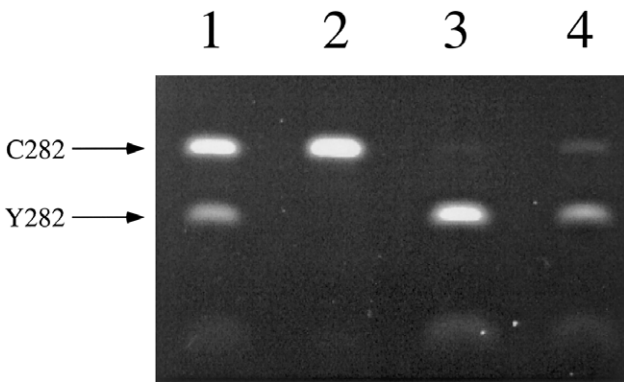

Figure 1 PCR amplification and *Sna*BI digestion of DNA (Jazwinska et al. 1996) from individuals referred for HH testing. Lane 1, C282Y carrier. Lane 2, Normal homozygote. Lane 3, C282Y homozygote. Lane 4, Individual with an anomalous pattern with trace amounts of undigested PCR product. All 25-μl PCR reactions were performed in parallel, with use of 150 ng DNA template and 1.25 U PLATINUM®*Taq* (GIBCO-BRL), in 20 mM Tris-HCl (pH 8.4), 50 mM KCl, and 1.5 mM $MgCl_2$. PCR conditions were as follows: 94°C for 2 min; then 30 cycles at 94°C for 30 s, 65°C for 30 s, and 72°C for 30 s; and a final extension at 72°C for 10 min, by means of a GeneAmp PCR System 9600 (Perkin-Elmer). PCR products were digested with *Sna*BI at 37°C for 3 h and were resolved by use of 1.5% agarose gel.

cently, we have identified anomalous results in some individuals while screening for the presence of the C282Y mutation. We initially identified eight individuals, seven of whom were unrelated, who appeared to be C282Y homozygotes with trace amounts of undigested DNA (fig. 1). It was assumed that these individuals were homozygotes with some form of sample contamination. Clinical histories of these individuals did not include previous blood transfusion or tissue transplantation. Increased amounts of restriction enzyme and incubation time, as well as resampling of these individuals, did not resolve the anomalous results. An increase in the stringency of the PCR conditions, achieved either by increasing the annealing temperature or by decreasing the amount of genomic template, reduced the amount of amplified normal product to generate a C282Y homozygote pattern (results not shown). Biochemical data on serum iron levels, serum ferritin levels, and transferrin saturation were available for two of these individuals—a 35-year-old man and a 71-year-old woman—and values were below the affected range. In one particular family, two sibs showed this pattern, yet, when their parents were tested, only the mother was found to be a C282Y carrier. In this instance, a combination of two independent incidents of nonpaternity and sample contamination would be required to explain the results.

We reanalyzed these cases, using two different approaches: (1) the Baty et al. (1998) amplification refractory mutation system (ARMS), which includes an alternative reverse primer with the Feder et al. (1996) forward primer, and (2) a modification of the previous protocol (Jazwinska et al. 1996) to incorporate two alternative primers that flank the Feder et al. primer sites (fig. 2). With both techniques, we found that all eight samples gave a clear carrier pattern for C282Y (results not shown). In all cases, sequencing across the Feder et al. primer sites revealed a G→A substitution at nucleotide position 5569 (GenBank accession number Z92910) on the non-C282Y allele. This sequence variant is located in intron 4, 5 bases from the 3′ terminus of the reverse-primer site identified by Feder et al., and is not predicted to disrupt normal hemochromatosis gene (HFE) splicing (fig. 2). In all eight samples, this mutation was seen on a non-C282Y, non-H63D chromosome. PCR using this reverse primer can result in dramatically reduced amplification of the polymorphic allele, such that a C282Y carrier can appear to be a C282Y homozygote. The relative intensity of the non-C282Y PCR product is inversely proportional to the stringency of the PCR conditions. Sequencing of both alleles in the eight individuals in the present study allowed clear assignment of carrier, rather than affected, status.

The 5569 G→A substitution introduces an *Mse*I restriction site. We used this enzyme, in conjunction with the Feder et al. (1996) forward primer and the HCS:

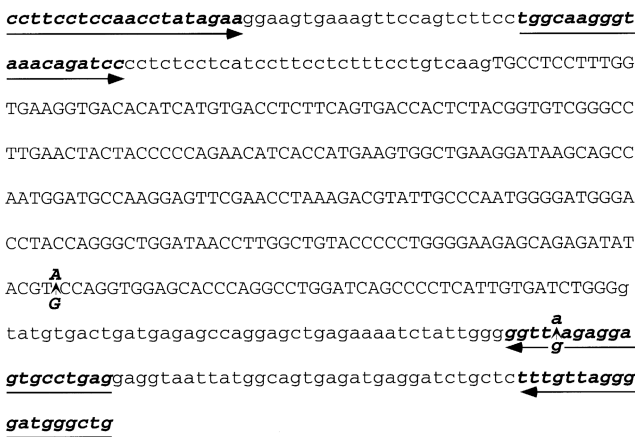

Figure 2 DNA sequence of HFE exon 4, with flanking introns. The relative locations of the C282Y mutation (G→A) in exon 4 and the 5569 G→A (892+48 G→A) polymorphism in intron 4 are shown. Sequences and locations are highlighted for Feder et al. (1996) forward and reverse primers and for the two alternative flanking primers (HCS:F and HCS:R) that were used to amplify this region. Conditions for PCR with HCS:F and HCS:R followed those outlined for the Feder et al. primer set (fig. 1), with an annealing temperature of 55°C. The 486-bp HCS PCR product was cleaved into 320- and 166-bp fragments by *Sna*BI in the presence of the C282Y mutation. The intronic polymorphism was confirmed, by sequencing and by *Mse*I digestion of HCS PCR products, in all samples that showed an anomalous *Sna*BI digestion pattern by use of Feder et al. (1996) PCR products.

R primer, to screen 48 individuals (45 of whom were unrelated) whom we had previously identified as C282Y homozygotes. In this group, we identified one additional unrelated individual with the C282Y mutation and the 5569 G→A polymorphism. Closer examination of the assay on the basis of which the previous diagnosis was made in this individual revealed an extremely faint normal band that had been interpreted to result from partial digestion. In total, therefore, the polymorphism has been found in 8 of 202 unrelated individuals who were referred for HH testing. An estimate of the allele frequency can be made on the basis of the C282Y carrier frequency. We found these 8 polymorphism carriers among a total of 43 unrelated C282Y (non-H63D) carriers. Our estimated population frequency of this allele is, therefore, 8/43 (=.186). Consequently, in our population, this polymorphism had the potential to result in ~19% of C282Y heterozygotes being misidentified as homozygotes.

We identified the 8 polymorphism carriers, in addition to 44 unrelated C282Y homozygotes, from our total sample of 202 unrelated individuals referred for testing. If the assumption of homozygosity, along with access to parental genotypes, had been made in all individuals with the polymorphism, as well as in those with the homozygosity, this would have led to an estimate of ~8/52 (=.154) for nonparentage, of which half of these cases, or 8%, would have been assumed to result from nonpaternity. The frequency of this polymorphism is high enough to warrant concern that the interpretation of homozygosity in these cases will result in an overestimate of the C282Y-allele frequency, a misdiagnosis of this condition, and an incorrect assumption of nonpaternity in some families. In our hands, the polymorphism promoted misinterpretation of a restriction-digestion–based assay, but any form of analysis (including allele-specific oligonucleotide hybridization, ARMS, or direct sequencing) that incorporates the Feder et al. (1996) reverse primer is equally prone to misdiagnosis. It is recommended that all laboratories using the Feder et al. reverse primer to test for the C282Y mutation confirm C282Y-homozygote results by using a flanking primer set and *Mse*I digestion.

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Electronic-Database Information

Accession numbers and URLs for data in this article are as follows:

GenBank, http://www.ncbi.nlm.nih.gov/Web/Genbank (for Z92910)
Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim (for HH [MIM 235200])

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No Mutations in the Coding Region of the *PRKCG* Gene in Three Families with Retinitis Pigmentosa Linked to the *RP11* Locus on Chromosome 19q

To the Editor:

Retinitis pigmentosa (RP) and allied degenerations of the retina are genetically heterogeneous, with well over 50 loci implicated so far through gene identifications or linkage-based chromosomal assignments. Among these genes, the dominantly inherited *RP11* locus (MIM 600138) on chromosome 19q is noteworthy because some carriers develop RP that is symptomatic at age <20 years, whereas others are asymptomatic and show no funduscopic or electroretinographic signs of disease even at age >70 years (Berson et al. 1969; Berson and Simonoff 1979; Evans et al. 1995; Nakazawa et al. 1996; McGee et al. 1997). On the basis of its chromosomal