Detection of the C282Y and H63D Polymorphisms Associated With Hereditary Hemochromatosis Using the ABI 7500 FAST Real Time PCR Platform

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Abstract: Classic hereditary hemochromatosis is an autosomal recessive disorder characterized by iron overload and sequence variants in the HFE gene. The HFE gene is located at 6p21.3 and contains 2 common single nucleotide polymorphisms (SNPs) C282Y and H63D, which are routinely tested for in the molecular diagnostics laboratory. In this study, we used DNA samples from 59 patients in which clinicians wanted to confirm or rule-out hereditary hemochromatosis that had been previously tested for the HFE SNPs using a polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) assay and the ABI 7700 real time PCR assay with a MGB Eclipse ASR Probe system. The new assay used TAQman SNP Genotyping Assays, which were performed on the ABI 7500 FAST real time PCR platform. Allelic discrimination was determined during a postamplification plate read. Of the 59 samples genotyped, 7 were homozygous for C282Y, 6 were heterozygous for C282Y, 9 were homozygous for H63D, 10 were heterozygous for H63D, 6 were compound heterozygotes, and 20 were wild type. With the exception of one sample that was indeterminate by the TAQman SNP Genotyping Assay, all others showed 100% concordance between the 3 assays. The one indeterminate sample was heterozygous for C282Y by the PCR-RFLP and ABI 7700 real time PCR assays, but there was an insufficient quantity of DNA to perform the TAQman SNP Genotyping Assay. Our study suggests that the ABI 7500 FAST TAQman SNP Genotyping Assay is comparable with the PCR-RFLP and ABI 7700 real time PCR methods in detecting and characterizing these 2 HFE SNPs. Improved software and thermocycling capabilities have resulted in a very robust TAQman assay with the advantage of a much improved turn-around-time and throughput.

Key Words: hereditary hemochromatosis, HFE, TAQman, ABI 7500

(MS) 2006:000–000)

MATERIALS AND METHODS

Samples

In this study, we used DNA samples from 59 patients who had been previously tested for the HFE C282Y and H63D SNPs using a PCR-restriction fragment length polymorphism (PCR-RFLP) assay and the ABI 7700 real time PCR assay with a MGB Eclipse ASR Probe system. Clinicians desired to confirm or rule-out hereditary hemochromatosis in these patients (on the basis of iron indices, family history, and/or clinical symptoms). DNA from all samples was isolated from whole blood using the PureGene Kit (Gentra, Inc, Minneapolis, MN).
The PCR-RFLP assay uses restriction enzyme digests to detect the difference between normal and SNP sequences for each mutation. For each patient sample, relevant portions of exons 2 (H63D locus) and 4 (C282Y locus) were amplified and restriction digested with NlaIII and RsaI, respectively, according to the method of Feder et al. The H63D mutation destroys a NlaIII site and the C282Y mutation creates a RsaI site. The digested products were electrophoresed on a 3% 3:1 Agarose gel (Amresco, Solon, OH) and visualized using ethidium bromide (Fig. 1).

**ABI 7700 Real Time PCR**

The ABI 7700 real time PCR assay with a MGB Eclipse ASR Probe system (Epoch Biosciences, Bothell, WA) was performed in transparent 96-well plates (PerkinElmer, Boston, MA) as follows. For each locus, approximately 10 to 20 ng of DNA was amplified in a 25 μL reaction containing 1 × PCR Buffer, 1 × primer mix, 1 × probe mix and 2 units Hotstart Taq (Sigma, St Louis, MO). Probes and primers for normal and SNP sequences were combined into a single reaction volume for each HFE mutation. Cycling conditions were as follows: 1 cycle of 50°C, 2 min/95°C, 2 minutes; 45 cycles of 95°C, 5 s/56°C, 20 s/72°C, 20 seconds; 1 melt cycle of 95°C, 15 s/40°C, 15 seconds; a 5-minute ramp-up to 80°C and a 15-second hold at 80°C. Data were collected during the thermocycling at the annealing step only and during the 5 minutes ramp-up to 80°C in the melt cycle. Multicomponent data were exported from the ABI 7700 as a .txt file. These data were then imported into the Eclipse Melt Macro supplied by EPOCH. The program plots the melt curve data and automatically calculates the identity of the alleles based on the input primer and probe data (Fig. 2). Table 1 shows the primers and probes used for the ABI 7700.

![Figure 1. PCR-RFLP results.](image1)

![Figure 2. ABI 7700 real time PCR assay results.](image2)
The new TaqMan assay on the ABI 7500 used 2 predesigned ABI Assays-on-demand (www.appliedbiosystems.com) containing primers and fluorescently labeled (FAM and VIC) MGB probes (20°C) for detection of the SNPs from the National Cancer Institute’s SNP500 database (http://snp500cancer.nci.nih.gov/home.cfm). Assays were run with the predesigned primer and probe SNP Genotyping Assays: C_1085595 (C282Y) and C_1085600 (H63D) using ABI 2°C fast universal master mix, 10 to 20 ng of genomic DNA in a total reaction volume of 10 μL using the default fast cycling conditions in transparent 96-well plates. The completion time is about 40 minutes. A postamplification plate read was used for allelic discrimination using the SDS software supplied with the instrument (Fig. 3).

**RESULTS**

Of 59 samples, 7 were homozygous for C282Y, 6 were heterozygous for C282Y, 9 were homozygous for H63D, 10 were heterozygous for H63D, 6 were compound heterozygotes, and 20 were wild type by all 3 assays (Table 2). One sample was heterozygous for C282Y by the PCR-RFLP and ABI 7700 real time PCR assays, but there was an insufficient quantity of DNA to perform the TaqMan SNP Genotyping Assay.

**DISCUSSION**

Classic hemochromatosis (HFE gene mutations) is one of at least 4 main types of hereditary hemochromatosis involving a number of genes and proteins involved in iron regulation within the body. Symptoms of hemochromatosis in the classic form are most commonly seen in C282Y homozygotes and less frequently in C282Y/H63D compound heterozygotes. Recent surveys involving HFE genotyping of general populations found that most C282Y homozygotes had incomplete penetrance with no clinical symptoms. This study raises the question of the value and cost-effectiveness of screening the general population. However, the ongoing Hemochromatosis...
and Iron Overload Screening (HEIRS) Study found that within the white participants 64% of men with adjusted transferrin saturation (TS) \( \geq 50\% \) had a HFE polymorphism and 63% of women with adjusted TS value \( \geq 45\% \) had a HFE polymorphism. They also showed that the positive predictive value for prediction of HFE gene polymorphism increased as the adjusted TS threshold increased.\(^6\) These results from the HEIRS study will be important in defining the screening guidelines in patients with increased iron indices for use in clinical practice.

The recently identified peptide hepcidin is believed to be the regulator that controls iron absorption and release by inducing the internalization and degradation of ferroportin, a major transmembrane iron exporter protein of macrophages and enterocytes. The function of the HFE gene in iron metabolism is now characterized by the “hepcidin model” hypothesis. Hepcidin is synthesized in the liver. Data suggest that HFE along with transferrin receptors 1 and 2 play a role in the iron-sensing pathway of hepatocytes, which subsequently regulates hepcidin synthesis. It is still unclear how HFE acts in this iron-sensing pathway, but hepcidin urine concentrations negatively correlate with the severity of hemochromatosis.\(^3\) Eventually, hepcidin urine and plasma assays will probably play a role in screening patients for iron overload diseases; however, these assays are not yet widely available. Currently, when iron overload is suspected (hyperferritinemia and/or increased TS are found) and acquired causes have been excluded, diagnosis of hereditary causes via molecular pathology techniques is considered.

In our study, patients were initially screened because clinicians wanted to confirm or rule-out classic hereditary hemochromatosis. With the exception of one sample that was indeterminate by the TAQman SNP Genotyping Assay, all others showed 100% concordance between assays. Our study suggests that the ABI 7500 FAST TAQman SNP Genotyping Assay is comparable with the PCR-RFLP and ABI 7700 real time PCR methods in detecting and characterizing these 2 HFE SNPs. Improved software and thermocycling capabilities have resulted in a very robust TAQman assay that has decreased analytical turn around times as compared with the ABI 7700 and gel-based PCR-RFLP assays. In addition, the 96-well plate format allows for a higher throughput than the gel-based assay.

### REFERENCES


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| Table 2. Results Summary of the ABI 7500 FAST TAQman SNP Genotyping Assay |
|---------------------------------|-----------------|-----------------|-----------------|
| ABI 7500 TAQman | RCR-RFLP | ABI 7700 |
| C282Y homozygous | 7 | 7 | 7 |
| C282Y heterozygous | 6* | 7 | 7 |
| H63D homozygous | 9 | 9 | 9 |
| H63D heterozygous | 10 | 10 | 10 |
| Compound heterozygotes | 6 | 6 | 6 |
| Wild type | 20 | 20 | 20 |
| Total* | 58* | 59 | 59 |

*One indeterminate sample on the TAQman SNP Genotyping Assay (due to insufficient quantity of DNA), which was heterozygous for C282Y by the PCR-RFLP and ABI 7700 real time PCR assays.
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