

Genotyping the HFE gene by melting point analysis with the LightCycler system: Pros and cons

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Abstract

The assay that combines rapid-cycle PCR with allele-specific fluorescent probe melting profiles performed on the Roche Diagnostics LightCycler is commonly employed for genotyping the HFE gene.

We report three illustrative cases of the pros and cons of this method. In two cases, atypical melting curves allows the identification of new DNA substitutions in the HFE gene, whereas, in the third case, a typical melting curve of c.845G>A mutation (C282Y) homozygosity overlooks a nucleotide change and promotes misdiagnosis of HH.

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Introduction

Hereditary hemochromatosis (HH) is a common inherited disorder of iron metabolism. There are six forms of HH associated with different genes and different patterns of inheritance. They include a main form, which is linked to the *HFE* gene and is called HH type 1, and five rare forms [1].

HH type I, which occurs predominantly in populations of Northwestern European descent (with a prevalence of 3–8 in 1000), is characterized by excessive iron absorption, leading progressively to the destruction of different organs, notably liver, pancreas and heart [2].

A number of different mutations have been identified in this gene worldwide, and one of them (c.845G > A) is present in a homozygous state in 80 to 95% of HH patients. The two other most common susceptibility factors of the *HFE* gene are

associated with milder forms of HH: c.187C > G (H63D) and c.193 A > T (S65C) [3,4].

Currently, there are a number of methods available for genotyping these mutations, the assay that combines rapid-cycle PCR with allele-specific fluorescent probe melting profiles being one of the most commonly employed. This single-tube analysis is performed on the Roche Diagnostics LightCycler, a microvolume fluorimeter integrated with a thermal cycler. Continuous acquisition of fluorescence data during a melting curve analysis at completion of PCR allows the detection of mutations given the loss of fluorescence in an allele-specific manner. By plotting melting peaks, the different alleles are readily distinguishable [5]. This melting analysis identifies single-base changes in PCR products with a sensitivity and specificity comparable or superior to conventional mutation scanning methods [6].

We analyzed genomic DNA from more than 3500 samples, and we report three illustrative cases: (i) in two cases, atypical melting curves allow the identification of new DNA substitutions in the HFE gene, and (ii) in the third case, a typical melting

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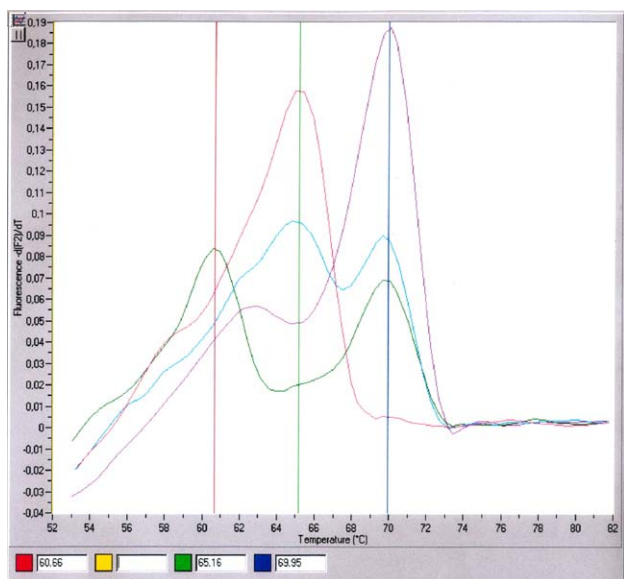


Fig. 1. LightCycler-PCR melting curve analysis. Red: wild type at position 282°C/C). Violet: homozygous for the C282Y mutation (Y/Y). Blue: heterozygous for the C282Y mutation (C/Y). Green: C282Y heterozygous and V284M heterozygous.

curve of c.845G > A homozygosity overlooks a nucleotide change and promotes misdiagnosis of HH.

Methods

The presence of three mutations of the HFE gene, c.845G > A, c.187C > G and c.193 A > T, was tested in the LightCycler™ (Roche Diagnostics GmbH, Mannheim, Germany). The site of the c.845G > A substitution was amplified with primers 282F (5'-TGG CAA GGG TAA ACA GAT CC-3') and 282R (5'-CTC AGG CAC TCC TCT CAA CC-3'). Amplification of the c.187C > G and c.193 A > T variant fragment was performed with primers 63F (5'-GCT CTG TCT CCA GGT TCA CAC TC-3') and 63R (5'-CCC TCT CCA CAT ACC CTT GC-3').

The cycle conditions were as follows: denaturation of the template DNA for 1 cycle of 95°C for 10 min, programmed temperature transition rate of 20°C/s and amplification of the target DNA for 45 cycles of 95°C for 10 s, 55°C for 10 s (each with a temperature transition rate of 20°C/s) and 72°C for 18 s (20°C/s).

The melting curve analysis was performed in 1 cycle of 95°C for 5 s and 45°C for 15 s, each with a temperature transition rate of 20°C/s and then ramping to 85°C at 0.1°C/s.

The c.187C > G was then monitored with the 5'-LC-Red705-labeled anchor (5'-CTG GTA TCC ACG TAG CCC AAA GCT TCA A-3') and the 3'-fluorescein-labeled sensor (5'-GG CGA CTC TCA TGA TCA TAG AAC ACG AAC A-3') probe. The c.193 A > T variant is detected in the same experiment as the c.187C > G change because it is located only six nucleotides away.

The c.845G > A transition at codon 282 is simultaneously analyzed with the 3'-fluorescein-labeled sensor (5'-GAG ATA TAC GTG CCA GGT GGA GCA C-3') and the 5'-LC-Red640-

labeled anchor probe (5'-AGG CCT GGA TCA GCC CCT CAT TGT-3').

Results

Case 1

We report a 39-year-old man in whom melting point analysis revealed an unusual curve progression. The melting points (T_M) of PCR products that are hybridized with probes corresponding to the HFE wild-type allele and the c.845G > A allele are 69°C ($\pm 0.30^\circ\text{C}$) and 65°C ($\pm 0.25^\circ\text{C}$), respectively. The patient's specimen showed a T_M of 69°C corresponding to that of the wild-type allele and an atypical T_M of 60.5°C (Fig. 1).

To sequence the region of the HFE gene containing the c.845G > A mutation, we performed a standard PCR using primers 282F and 282R described above, which led to amplification of a 390-bp fragment. The amplicons were purified, subjected to the Big Dye Terminator Cycle Sequencing protocol and analyzed on the ABI PRISM 310 genetic analyzer (Applied Biosystems). Sequences revealed the presence of two G > A heterozygous substitutions in position 845 (C282Y) and position 850 (V284M) (Fig. 2).

The study of additional members of the patient's family revealed that this last substitution is located in *cis*, two codons away from the c.845G > A mutation.

Case 2

We describe a 50-year-old man in whom the melting curve analysis yielded one peak corresponding to an c.187C > G wild-type allele (65°C \pm 0.35) and an allele with an unexpected melting peak at 62.6°C (Fig. 3). This peak does not correspond to the c.193 A > T because this mutation has a melting peak at 61.4°C \pm 0.35, or 3.6°C lower than the melting temperature of the c.187C > G wild-type allele [7] (Fig. 4).

ATACGTNCCAGNTGGAG

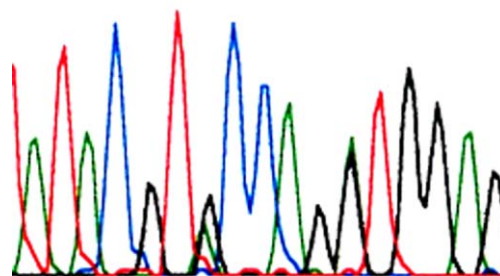


Fig. 2. Sequence of c.845 G > A mutation (C282Y) and c.850 G > A mutation (V284M).

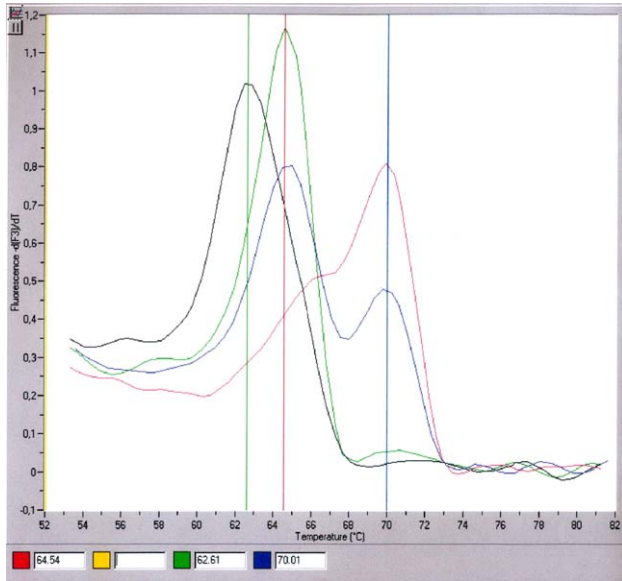


Fig. 3. LightCycler-PCR melting curve analysis. Green: wild type for the H63D mutation (H/H). Red: homozygous for the H63D mutation (D/D). Blue: heterozygous for the H63D mutation. Black: heterozygous for the L57L change. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Subsequent sequencing of the corresponding PCR fragment revealed a G > C substitution at position 171 (c.171G > C), which causes a silent change (Leu 57 Leu) (Fig. 5).

Case 3

In the DNA sample of a 60-year-old patient, melting point analysis revealed a very unusual pattern: that of a c.845G > A

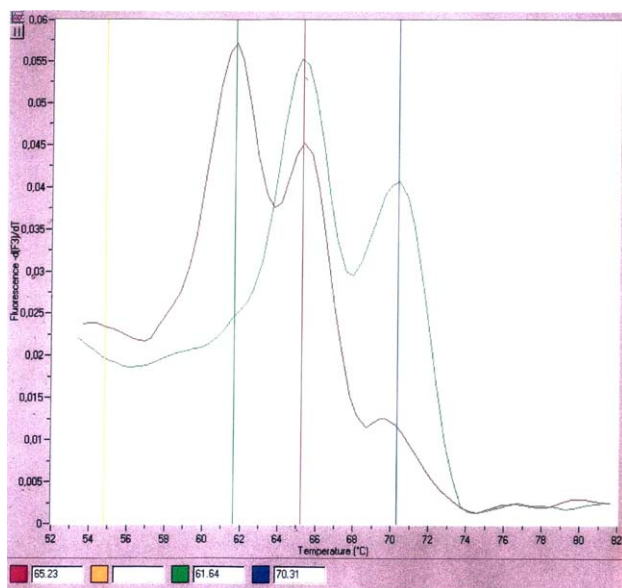


Fig. 4. LightCycler-PCR melting curve analysis. Red: heterozygous for the S65C mutation. Green: heterozygous for the H63D mutation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

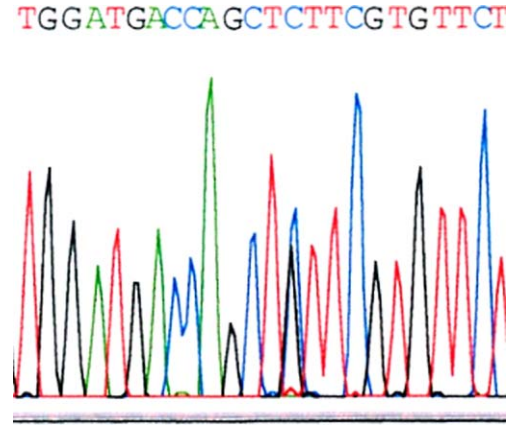


Fig. 5. Sequence of c.171 G > C change (L57L) in the HFE gene.

homozygote and of a c.187C > G heterozygote. We reanalyzed the sample using the classical PCR amplification followed by restriction digestion. The results indicated that the patient was a compound heterozygote c.845G > A/c.187C > G. The sequence of the region of the HFE gene containing the c.845G > A mutation revealed that, in addition to the c.845G > A mutation, there was a G > A change (located two nucleotides before the G > A substitution that causes the c.845G > A mutation), which causes a silent mutation (T281T) (Fig. 6). This situation in which two G > A changes are separated by only two nucleotides explains why the melting point analysis identified the sample as a c.845G > A homozygote.

Using classical PCR followed by restriction digestion, we reanalyzed all the c.845G > A homozygotes identified with the allele-specific fluorescent probe melting profiles ($n = 106$) to determine whether other misdiagnoses had occurred. Fortunately, the two methods gave identical genotypes in all cases.

Discussion

The main advantage of genotyping HFE mutations on the LightCycler with fluorescent hybridization probes is that the PCR and detection are simultaneously performed in the same capillary tube with no post-PCR manipulation with the result

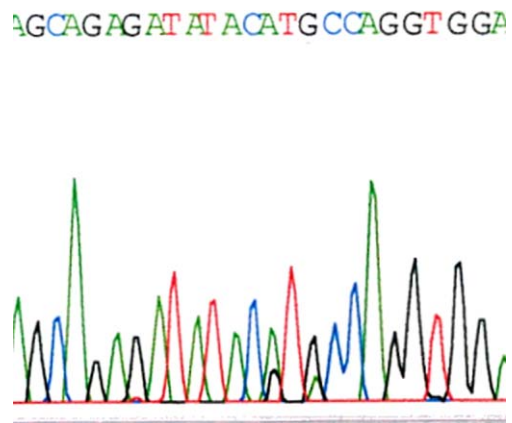


Fig. 6. Sequence of c.843 G > A mutation (T281T) and c.845 G > A mutation (C282Y).

that the method is very fast and there is less opportunity for error due to sample manipulation. This assay is suitable for molecular diagnostic routine laboratories, where a large number of samples must be processed [7,8].

To date, we have determined the HFE genotype of more than 3500 individuals, and variations of the known melting profiles were observed in two unrelated cases. DNA sequencing or RFLP analysis was performed to unequivocally assign the correct HFE genotype. The unusual melting curve profile, thus, allowed the identification of novel substitutions in the HFE gene (V284M and L57L). This lends support to the validity of this method for genotyping the HFE gene. Only one case has been reported [9] in which a variant pattern of the HFE-LightCycler melting profiles preventing allelic discrimination is not caused by the primary sequence of the patient DNA per se.

Case 3 illustrates an example of misdiagnosis. The pattern observed corresponds to an individual homozygous for the c.845G > A mutation and at the same time heterozygous for the c.187C > G mutation in the HFE gene. Although it has been claimed that the two mutations are mutually exclusive, there are two reports describing a proband with homozygous mutation for c.845G > A and a heterozygous mutation at the c.187C > G site [10,11]. In our experience, this is the first case in which the c.845G > A and the c.187C > G mutations existed on the same DNA strand in accordance with the melting point analysis. Sequencing the region of the HFE gene containing the c.845G > A mutation performed to confirm this rare finding revealed that, in addition to the c.845G > A mutation, there was a G > A change (located two nucleotides before the G > A substitution that causes the c.845G > A mutation) which causes a silent mutation (T281T). The presence of two almost consecutive G > A changes causes the melting point analysis to erroneously identify the sample as a c.845G > A homozygote. To determine whether other misdiagnoses had occurred due to the T281T silent mutation in the HFE gene, we analyzed, using classical PCR followed by restriction digestion, all the c.845G > A homozygotes identified, in our laboratory, with the allele-specific fluorescent probe melting profiles ($n = 106$). In all cases, the two methods gave identical genotypes, suggesting that this silent mutation is not frequent.

Other situations that give a false-positive c.845G > A result owing to the presence of a single nucleotide polymorphism that interfered with primer binding in the PCR assay were described [12,13]. The main importance of these two polymorphisms (IVS4+48 g > a and IVS3-48c > g) is that they may lead to

misdiagnosis of heterozygotes for the c.845G > A mutation as homozygotes. In both cases, the problem may be resolved by using alternate primers while the situation exemplified in case 3 may be clarified using classical PCR followed by restriction digestion.

Acknowledgments

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