Young Woo Park, Seng-Lai Tan and Michael G. Katze
University of Washington
Seattle, WA, USA

Rapid Genetic Screening for Hemochromatosis Using Automated SSCP-Based Capillary Electrophoresis (SSCP-CE)

ABSTRACT

Hereditary hemochromatosis (HHC) represents an autosomal recessive disease in which increased iron absorption causes iron overload and irreversible tissue damage. The recently detected association between two point mutations in the HFE gene on chromosome 6p and HHC has made it possible to screen for the disease before the onset of irreversible tissue damage. Conventional genetic testing is based on restriction fragment-length polymorphisms (RFLP) using two endonuclease recognition sites in codon 63 or 282, respectively. In this study, we have adapted single-strand conformation polymorphism analysis for capillary electrophoresis (SSCP-CE) to detect homozygote or heterozygote point mutations. Two HFE gene fragments spanning codons 63 and 282 were amplified by a duplex PCR using genomic DNA from peripheral blood or from tissue sections of paraffin-embedded liver biopsies as template. Thereby, rapid genotyping of both HFE mutations was achieved with a single PCR, omitting the need of further analysis by restriction digest. Eighty-five patients with liver disease and/or suspected iron overload were genotyped using SSCP-CE, and all results were verified by conventional RFLP analysis. In summary, SSCP-CE proved to be a reliable, cost-effective, sensitive and rapid method for genotyping HFE mutations. This method will further facilitate high-throughput genetic screening using capillary array electrophoretic devices.

INTRODUCTION

Hereditary hemochromatosis (HHC) is an autosomal recessive disease in which increased iron absorption causes iron overload and progressive tissue damage. Clinical manifestations include diabetes, cardiomyopathy, hypogonadotropic hypogonadism, arthritis and liver cirrhosis, which might ultimately lead to hepatocellular cancer. HHC represents one of the most common genetic diseases in Northern Europe and the UK, with an estimated carrier frequency between 1:8 and 1:10 (12). Conventional diagnosis of the disease in its symptomatic stage is based on a combination of clinical, biochemical and histological parameters (4), as described in detail in Materials and Methods.

However, these parameters lack specificity and fail to detect HHC in its asymptomatic stage, which is important since iron removal by phlebotomy is an effective means to prevent irreversible tissue damage. Importantly, an association between HHC and mutations in the HFE gene, a major histocompatibility complex class I-like gene located on chromosome 6p, has been described recently (5,7,8). The majority of patients turned out to have the same point mutation changing a cysteine at amino acid position 282 to tyrosine (C282Y). This mutation is most prevalent in Northern Europe, North America and Australia and was detected in 91% of HHC patients in the UK (6) but only in 64% of Italian patients (3). A second mutation, changing the histidine at position 63 to aspartatic acid (H63D), was detected in a minority of patients (2,5,8). However, involvement of this mutation in the full clinical manifestation of HHC has not yet been formally confirmed. Different clinical phenotypes have been associated with different types of mutations (C282Y homozygosity vs. C282Y and H63D compound heterozygosity) and the most common ancestral haplotype, which is linked to HLA-A3 on chromosome 6p (1,9,10).

In summary, these studies have provided a reliable genetic tool to detect HHC early during its presymptomatic course and thus prevent all clinical manifestations. Therefore, an easy, cost-effective test for the detection of HFE mutations will facilitate population-based screening. Here, we describe a protocol that adapts single-strand conformation polymorphism (SSCP) analysis for capillary electrophoresis and that is suitable for large-scale, cost-effective genetic testing.
MATERIALS AND METHODS

Patients and Samples

Seventy-nine patients (male:female = 47:32) ranging from 24–78 years of age who were referred to the University of Regensburg Medical School’s Department of Gastroenterology to evaluate liver disease and/or suspected iron overload were included into this study. Further, six patients were included with known HHC diagnosed on the basis of the following parameters:

(i) increased transferrin saturation (repeatedly >50% at fast) and increased serum ferritin levels;
(ii) hepatocellular hemosiderin deposits of grade III-IV;
(iii) hepatic iron index (HII) >2 and/or total amount of iron removed (IR) >5 g in men, > 3 g in women; and
(iv) exclusion of iron-loading anemias or history of blood transfusion.

After informed consent of the patients, peripheral blood specimens and, in some cases, liver fine-needle biopsies were obtained. Genomic DNA specimens were prepared either from 200 µL blood or from 5 consecutive 10-µm tissue sections of paraffin-embedded liver biopsates using the QIAamp Blood Kit precisely following the manufacturer’s protocol (Qiagen GmbH, Hilden, Germany). In the case of liver biopsates, the tissue sections were collected in a 1.5-mL reaction vial, incubated with 200 µL proteinase K (50 µg/mL) in 0.5% sodium dodecyl sulfate (SDS) at 55°C overnight and further processed with the QIAamp Blood Kit. Finally, the DNA specimens were dissolved in 200 µL H₂O.

Duplex PCR

One microliter dNTPs (10 mM each), 2 µL primer (25 pM each), 5 µL 10-fold Taq buffer (1.5 mmol MgCl₂, final concentration) and 4 U recombinant Taq DNA polymerase (Amersham Pharmacia Biotech, Freiburg, Germany) were added to 5 µL template DNA and polymerase chain reaction (PCR)-amplified in a total reaction volume of 50 µL using the following reaction profile: 1 min at 94°C, 30 s at 62°C and 1 min at 72°C. For blood specimens, DNA concentrations were approximately 50 ng/µL; for paraffin sections, they varied from 10–30 ng/µL. Before the first cycle, the reaction mixture was denatured for 5 min at 94°C, and after the last cycle, extension was completed for 2 min at 72°C. All reactions were performed on a GeneAmp PCR System 9700 (PE Biosystems, Weiterstadt, Germany).

For analysis of the HFE codon 282, the FAM-labeled forward primer 5′-GCC CAA TGG GGA TGG GAC-3′ (matching nucleic acid residues 6648 to 6665) and the unlabeled reverse primer 5′-CCC AAT AGA TTT TCT CAG C-3′ (residues 6812 to 6794) were used; for analysis of the HFE codon 63, the unlabeled forward primer 5′-ACT ACC TCT TCA TGG GTG CC-3′ (residues 4667 to 4686) and the TAMRA-labeled reverse primer 5′-GCC ACA TCT GGC TTG AAA TT-3′ (residues 4828 to 4809) were used. These primers match to invariant regions of the HFE gene.

To purify the PCR amplification products, 50 µL of polyethylene glycol (PEG) solution (52.4 g PEG 8000, 40 mL M Na-acetate, pH 5.2, 1.32 mL 1 M MgCl₂) were added to the reaction mixture, vortex mixed, incubated for 20 min at room temperature, pelleted for 30 min at 3200 rpm with a Model 3757 Biofuge Rotor (Heraeus, Hanau, Germany) and washed with 100 µL ethanol. Finally, the dried pellet was dissolved in 20 µL H₂O.

Details of the clinical data will be published elsewhere.

Table 1. HFE Genotyping by SSCP-CE

<table>
<thead>
<tr>
<th>Genotype</th>
<th>C282Y</th>
<th>H63D</th>
</tr>
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<tbody>
<tr>
<td>WT/WT</td>
<td>54</td>
<td>70</td>
</tr>
<tr>
<td>WT/muta</td>
<td>14</td>
<td>14</td>
</tr>
<tr>
<td>mut/mut</td>
<td>17</td>
<td></td>
</tr>
<tr>
<td>n = 85</td>
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</table>

a Two of these patients were compound heterozygotes.
b This patient presented with severe systemic iron overload and the full clinical phenotype of hemochromatosis, including liver cirrhosis.

To determine the HFE genotype by conventional restriction fragment-length polymorphism (RFLP), the two PCR amplifications were performed...
separately. Half of the PEG-purified reaction product (10 µL) was digested overnight with 15 U of the appropriate restriction enzyme. SnaBI digest resulted in a 164-bp C282 wild-type (WT) fragment or, in the case of the C282Y mutation, two 72- and 92-bp fragments. BclI digest resulted in a 94- and 67-bp H63 fragment, respectively, or a single 161-bp mutated H63D fragment. Restriction fragments were electrophoresed on 5% polyacrylamide (PAA)/TBE gels and visualized with ethidium bromide.

**Capillary Electrophoresis**

One microliter of PEG-purified PCR product was added to 12 µL Template Suppression Reagent (TSR; PE Biosystems), 0.5 µL 0.3 N NaOH and 0.5 µL HD-400-ROX standard (PE Biosystems), mixed, incubated for 2 min at 95°C and immediately cooled on ice for 10 min. Separation of PCR products was performed on an ABI 310 Capillary Electrophoresis Device using the GENESCAN™ Data Collection (Version 1.0.4) and Analysis Software (Version 2.1) and the GS-Template 1-mL Filter A module (all from PE Biosystems). A 47-cm/50-µm green capillary was filled with 5% gene-scan-polymer/10% glycerol (PE Biosystems) in (i) 1× TBE buffer (codon 63 analysis) and (ii) 1× genetic analysis buffer with EDTA (codon 282 analysis). Additional parameters were: injection time 10 s; injection voltage 15 kV; electrophoresis voltage 13 kV; collection time 24 min; temperature 30°C; syringe pump time 180 s; and pre-injection time 0 s.

**RESULTS AND DISCUSSION**

Three representative electrophoretic profiles resulting from SSCP-capillary electrophoresis (CE) analysis of HFE codon 282 PCR fragments are shown in Figure 1A. PCR fragments are displayed in blue vs. the HD-400 ROX standard, which is displayed in red. Fragments from patients with two WT alleles ran significantly faster with respect to the standard than fragments from patients with homozygous C282Y mutations. Consistently, profiles from patients with heterozygous mutations revealed two peaks overlapping precisely with the WT and the mutated fragments, respectively. Identification of WT, heterozygote or homozygote patients was possible solely on the basis of the electrophoretic mobility of the fragments and was not dependent on the peak height. Thus, the SSCP profile allowed unambiguous genotyping even in the case of large differences in PCR efficiency. This was important because in a number of cases we had to use very small amounts of DNA extracted from paraffin-embedded liver biopsies, which resulted in significant differences in PCR performance and peak heights.

Characteristic profiles of SSCP were also observed when PCR fragments of the HFE codon 63 region were analyzed by CE (Figure 1B). Wild-type codon 63 PCR fragments (displayed in black) revealed significantly higher electrophoretic mobility than the fragment from a patient with a homozygous mutation. PCR products from patients with heterozygous mutations revealed two peaks. Again, unambiguous genotyping of HFE codon 63 was possible by determination of the fragment mobility in relation to the standard, independent of the relative peak height and

![Figure 1. SSCP of HFE codon 282 and HFE codon 63 mutations.](attachment:image.png)
PCR efficiency. However, due to the different buffer that was used for codon 63 analyses (TBE), the ROX-standard profile varied from the profile of the codon 282 analyses.

To test performance and reliability of SSCP-CE, we genotyped HFE codon 63 and 282 of 79 patients who were referred to the Department of Gastroenterology of our clinic for evaluation of liver disease and/or suspected iron overload. Further, six patients with known hemochromatosis were analyzed. Table 1 summarizes our results and shows that we detected 17 patients with homozygous C282Y mutations, 1 patient with a homozygous H63D mutation and 2 patients with compound heterozygous mutations. All of these patients revealed the clinical phenotype of hemochromatosis, in contrast to only 2 of the 54 patients with two WT C282 alleles.

Surprisingly, the genotype detected by SSCP-CE differed in three cases from the results obtained by conventional RFLP. Two patients classified by RFLP as C282Y heterozygotes tested homozygote by SSCP-CE, and 1 patient classified by RFLP as H63D heterozygote was determined WT on both alleles by SSCP-CE. Therefore, the PCR fragments were sequenced entirely, and the RFLP typing was repeated. In all three cases, the results obtained by SSCP-CE were confirmed. When we reanalyzed the original RFLP gels, we found that incomplete restriction digest in combination with poor PCR efficiency had compromised the interpretation of the banding pattern. Obviously, SSCP-CE does not require restriction digest and allows interpretation even at very low concentrations of PCR products and therefore represents a very robust method.

For the best performance, SSCP analysis of codon 63 fragments required 1× TBE buffer, in contrast to codon 282 analysis, which required 1× genetic analysis buffer (PE Biosystems). Therefore, we had to perform two consecutive runs of CE in our small-scale setup. Since only 1/20 of the entire PCR product was required for a single capillary electrophoretic run, it is easy to analyze two PCR aliquots in parallel on multiple micro-fabricated capillary array electrophoretic devices, which allow rapid high-throughput genetic testing (11). SSCP-CE has a number of further conceptual advantages over conventional RFLP analyses. It can be performed on fully automated robot systems on a very large scale, it does not involve restriction digest and is therefore cheaper, faster and more robust with respect to misinterpretation of partial restriction digests. In summary, our method will facilitate population-based screening programs when performed on capillary array electrophoretic devices.

REFERENCES


A.-K. Bosserhoff and S. Seegers contributed equally to this manuscript. Address correspondence to Dr. Reinhard Büttner, Institute for Pathology, University Hospital RWTH Aachen, D-52074 Aachen, Germany. Internet: buettner@pat.rwth-aachen.de

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A.-K. Bosserhoff, S. Seegers, C. Hellerbrand, J. Schölmerich and R. Büttner
University of Regensburg
Medical School
Regensburg, Germany