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Journal of Chromatography B, 754 (2001) 265–270

JOURNAL OF CHROMATOGRAPHY B

www.elsevier.com/locate/chromb

Short communication

High-performance liquid chromatography multiplex detection of two single nucleotide mutations associated with hereditary hemochromatosis

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Received 29 August 2000; received in revised form 16 November 2000; accepted 22 November 2000

Abstract

High-performance liquid chromatography (HPLC) has been applied to the multiplex detection of the two single nucleotide mutations commonly found in hereditary hemochromatosis (HH). HH is associated with a major G to A transition at position 845 (mutation Cys282Tyr) and a minor C to G transition at position 187 (mutation His63Asp) in the cDNA of the HFE gene. Two detection assays were developed based on HPLC analysis of restriction fragment length polymorphism (RFLP) or single nucleotide extension (SNE) products following multiplex PCR amplification. RFLP genotypes the two sites as dsDNA fragments of different lengths generated by restriction enzymes Rsa I/Bcl I. SNE extends primers 5′-adjacent to the sites of interest with a dideoxynucleotide triphosphate (ddNTP) to generate extended ssDNA. The identity of the added ddNTP reveals the identity of the original possible mutation site(s). Application of these methods with HPLC analysis provides simple and reliable genotyping for HH and can be applied to other single nucleotide polymorphism studies.

Keywords: Multiplex detection; Nucleotide mutations

1. Introduction

Hereditary hemochromatosis (HH) is one of the most common hereditary disorders with a prevalence of 2–5 in every 1000 Caucasian descendents [1]. HH is an autosomal recessive metabolic disorder resulting in excessive iron accumulated in various organs such as liver, pancreas and heart [2]. Without treatment, irreversible organ damage and dysfunction can develop, including cirrhosis, heart failure, diabetes, and impotence. Fortunately, hemochromatosis can be treated simply by removal of the iron burden by regular phlebotomy [1]. Early diagnosis, before the development of cirrhosis or any other irreversible organ damage occurs, is critical for an effective treatment. HH is believed to be associated with two single nucleotide transitions in the HFE gene. A major single nucleotide transition, G to A, at nucleotide 845 in cDNA (Genebank access No. U60319) is observed in 80–90% of the HH patients in northern European countries. To a lesser extend, a C to G transition at 187 may also responsible for iron-

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0378-4347/01/$ – see front matter
PII: S0378-4347(00)00607-1

overload [3,4]. These two changes in the HFE gene cause a cysteine (C) to tyrosine (Y) mutation at codon 282 (C282Y), and a histidine (H) to aspartic acid (D) mutation at codon 63 (H63D), respectively. The association of these two mutations and HH makes the early diagnosis of HH possible and feasible.

Restriction fragment length polymorphism (RFLP) analysis by gel electrophoresis is the most commonly used method for detection of HH-associated mutations [4–6] due to its low requirement in instrumentation. RFLP employs restriction enzymes that recognize either the wild-type loci or the mutant loci resulting in digested DNA fragments of substantially different sizes that can be differentiated corresponding to the genotypes. The major drawback of slab gel analysis is lack of automation and the use of mutagenic materials for DNA detection. High-performance liquid chromatography (HPLC) has distinct advantages over slab gel analysis of polymorphisms and has received considerable attention [7–9]. Recently, denaturing HPLC has been applied for the detection of the two common HH mutations [10], based on the temperature-modulated formation of a heteroduplex and the gradient-modulated separation of the heteroduplex from the homoduplex [11]. This method genotypes the two mutations in a single multiplex PCR followed by two separate denaturing HPLC analyses on samples with and without addition of wild-type DNA. Alternatively, single nucleotide extension (SNE) has been combined with HPLC [12] and other instrumentation including capillary electrophoresis [13,14] and mass spectrometry [15] for mutation detection. SNE requires an upstream or a downstream primer immediately 5′ to the possible mutation site. The primer is extended and terminated at the 3′-end, with the addition of one of the four dideoxynucleo-tide triphosphates (ddNTP), complimentary to the nucleotide at the possible mutation site [16].

Here, we have applied HPLC to the analysis of RFLP or SNE products for simultaneous HFE genotyping at codons 282 and 63. DNA was extracted from the blood samples of patients, amplified by PCR for the two sites of interest, and then treated either by restriction enzymes or by single nucleotide extension, with subsequent analysis by HPLC using ion-pairing conditions.

2. Methods and materials

2.1. Primer design

Primers chosen for PCR amplification of the C282Y and H63D loci were based on examination of the HFE gene, Genebank access No. Z92910. The two mutations are due to the transition of G to A at 6722 and C to G transition at 4762, respectively. Primers were evaluated by a free online program (www.williamstone.com/primers) and synthesized on an Expedite Nucleic Acid Synthesis System (PerSeptive Biosystems, Inc., Framingham, MA). For RFLP studies, primers were chosen so that a minimum number of restriction fragments, with considerable length differences, were generated after Rsa I/Bcl I treatment. For SNE studies, the choice of PCR primers is more flexible, as it is the SNE products, rather than the initial PCR products, which are finally analyzed by HPLC [14]. We chose different PCR primers for the two sample preparation methods to demonstrate that for SNE studies, primers used for amplification can also be used as primers for the extension. The 282-site was amplified as 132 bp by primers HR/SE21 for RFLP and 38 bp by primers SE16/SE21 for SNE. The 63-site was amplified as 90 bp by primers H2/SE23 for RFLP and 42 bp by primers SE18/SE21 for SNE. The sequences of primers are: HR (5′-CTCAGGCACTCCTCTCAACC-3′), SE16 (5′-TGGGTGCTCCACCTGG-3′), SE18 (5′-CCACACGGCGACTCTCAT-3′), SE21 (5′-GGGAAGAGCAGAGATATACGT-3′), SE23 (5′-ACCAGCTGTTCGTGTTCTATGAT-3′), and H2 (5′-GCCACATCTGGCTTGAAATT-3′).

2.2. DNA extraction and PCR amplification

Genomic DNA was extracted from human blood with the Puregene DNA Isolation Kit (Gentra Systems, Inc., Minneapolis, MN) following the manufacturer's protocol. PCR reactions were performed using the GeneAmp PCR system 9600 thermal reaction cycler (PE Biosystems, Foster City, CA). PCR was carried out in 20 μl total reaction volume containing 10 ng extracted DNA, 2 μl of 10X PCR reaction buffer, 0.4 μl of dNTP mix (Boehringer Mannheim Corp., Indianapolis, IN), 0.2 μl of BSA

(4 mg/ml), 2 μl of the 10 μM appropriate primers, and 1 unit of AmpliTaq Gold™ polymerase (PE Biosystems). For enzymatic digestion studies, HR/SE21 and H2/SE23 were added to PCR mix, while for SNE analysis, SE16/SE21 and SE18/SE23 were added to PCR mix. Cycling conditions were 96°C for 10 min, followed by 36 cycles of 94°C for 10 s, 60°C for 20 s, 72°C for 20 s, and a final extension step at 72°C for 7 min.

2.3. SNE reaction

Following PCR amplification, unincorporated primers and dNTPs were eliminated from the PCR product mixture by adding 1 unit of shrimp alkaline phosphatase and 5 unit of Exonuclease I (both from USB Corp., Cleveland, OH). The mix was incubated at 37°C for 30 min followed by heat inactivation of enzymes at 80°C for 15 min. SNE reaction was carried out in 20 μl total volume containing 2 μl of purified PCR products, 4 μl of 5X reaction buffer, 2 μl of the 10 μM primers SE16 and SE23, 1 μl of ddNTP mix (1 mM each, USB Corp.) and 0.2 μl AmpliTaq polymerase FS. The reaction buffer and the polymerase were from the ABI PRISM dye terminator cycle sequencing core kit (PE Biosystems). Primer extension reactions were performed with 1 min at 94°C, followed by 20 cycles of 30s at 92°C and 40s at 70°C with a decrease of 0.5°C/cycle. This is a general protocol to cover a range of annealing temperatures to allow several primers to be annealed and extended [13].

2.4. Restriction enzymatic digestion

To 15.5 μl of PCR product, 1 μl Reaction Buffer C (Promega, Madison, WI), 1 μl each of Rsa I (10 U/μl, Promega) and Bcl I (10 U/μl, GIBCO BRL, Gaithersburg, MD), 0.5 μl BSA (10 mg/ml, Promega) were combined and heated at 37°C for 30 min and at 50°C for 30 min.

2.5. HPLC analysis

Restriction digested products or SNE products were analyzed on a Transgenomic HPLC (Transgenomic, Inc., San Jose, CA) with a variable wavelength detector set at 260 nm. A DNASep™ column (Transgenomic, Inc.) was used to separate DNA fragments. The column temperature was set to 54°C, the flow rate was set at 0.8 ml/min. With a 96 well auto-sampler, 10 μl of crude reaction mixture was injected automatically into the HPLC. DNA fragments were eluted under ion-pairing conditions with a linear gradient mixture of Buffer A: 0.1 M TEAA (Transgenomic) and Buffer B: 0.1 M TEAA/25% acetonitrile. SNE products were eluted with a linear increase of 2% B per min over a period of 10 min, starting from 22% and ending with 42% solvent B. Restriction digested products were eluted starting from 25% B and ending with 42% B in 1 min followed by a linear increase of 2% B over a period of 10 min, starting from 42% and ending with 62% solvent B. The column was regenerated in 4 min following each analytical run.

3. Results and discussions

Genotyping of the two regions of the HFE gene containing the mutations C282Y and H63D was performed by PCR amplification of total genomic DNA and then followed by either of two sample preparation methods. For RFLP analysis, amplicons of 132 bp and 90 bp generated by primers HR/SE21 and H2/SE23 were treated by restriction enzymes Rsa I and Bcl I. As listed in Table 1 and demonstrated in Fig. 1, the G to A transition (C282Y) created a new Rsa I site while the C to T (H63D) transition eliminated the Bcl I site. The 132 bp amplicon was cut into 111 bp and 21 bp fragments for the mutant 282-loci, while the 90 bp amplicon was digested into 66 bp and 23 bp for the wild-type 63-loci. Under the current HPLC conditions, the short DNA fragments of 21 bp and 23 bp were indistinguishable as they co-eluted with the solvent front. Thus, genotyping of these two sites was determined by which of the four enzymatic digestion peaks, 132 bp, 111 bp, 90 bp, 66 bp, appeared in the HPLC analysis, as shown in Fig. 1.

For SNE studies, primers SE16/SE21 and SE18/SE23, directly upstream and downstream adjacent to the 282-site and the 63-site, respectively, were chosen to amplify the two sites of interest [14]. After PCR and post-PCR purification, SE16 and SE23 were added as primers for the extension reactions,

Table 1
Analysis of the enzymatic digestion of the PCR products for the 282-site and the 63-site for different genotypes[a]

Enzyme	Rsa I for the 282-site	Bcl I for the 63-site
Digestion site	5′-GT↓AC-3′	5′-TG↓ATCA-3′
PCR product	132 bp	90 bp
Wild-type	5′-GGCACG-3′	5′-ATG↓ATCAT-3′
	132 bp, not digested	Digested into 66 bp, (23 bp)
Mutant	5′-GG**T**↓ACG-3′	5′-AT**C**ATCAT-3′
	Digested into 111 bp, (21 bp)	90 bp, not digested

[a] The short DNA fragments of 21 bp and 23 bp were not resolved under the current conditions, thus were bracketed.

and the extended ssDNA was analyzed by HPLC. Fig. 2 presents the HPLC analysis of SNE products generated from four samples of known genotype. Peaks corresponding to primers SE16 and SE23 eluted at 4.2 min and 7.8 min, respectively. The SNE products from a normal or a mutant 282-loci resulted in a peak at close to 4.8 min or 5.6 min, respectively; while a normal or a mutant 63-loci products eluted at 8.0 min or 8.2 min, respectively. Under current conditions, each primer peak was followed by its normal product peak and then its mutant product peak. The positioning of the product peaks relative to primer peaks also aids in peak identification. The variation in elution time was in general less than 0.1

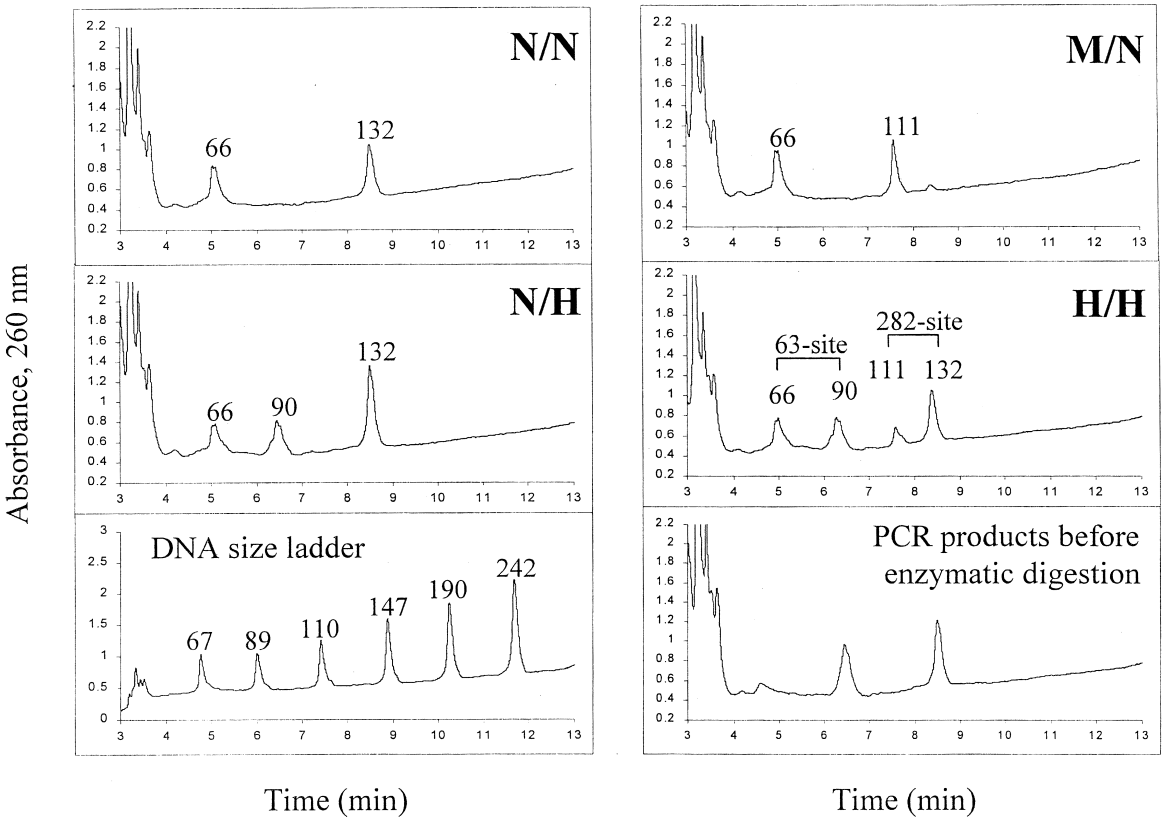

Fig. 1. HPLC chromatograms of multiplex detection of the two HH-related mutations in four genotypes, by RFLP analysis. Genotype is 282-site/63-site. H, heterozygote; M, homozygote mutant; N, homozygote normal.

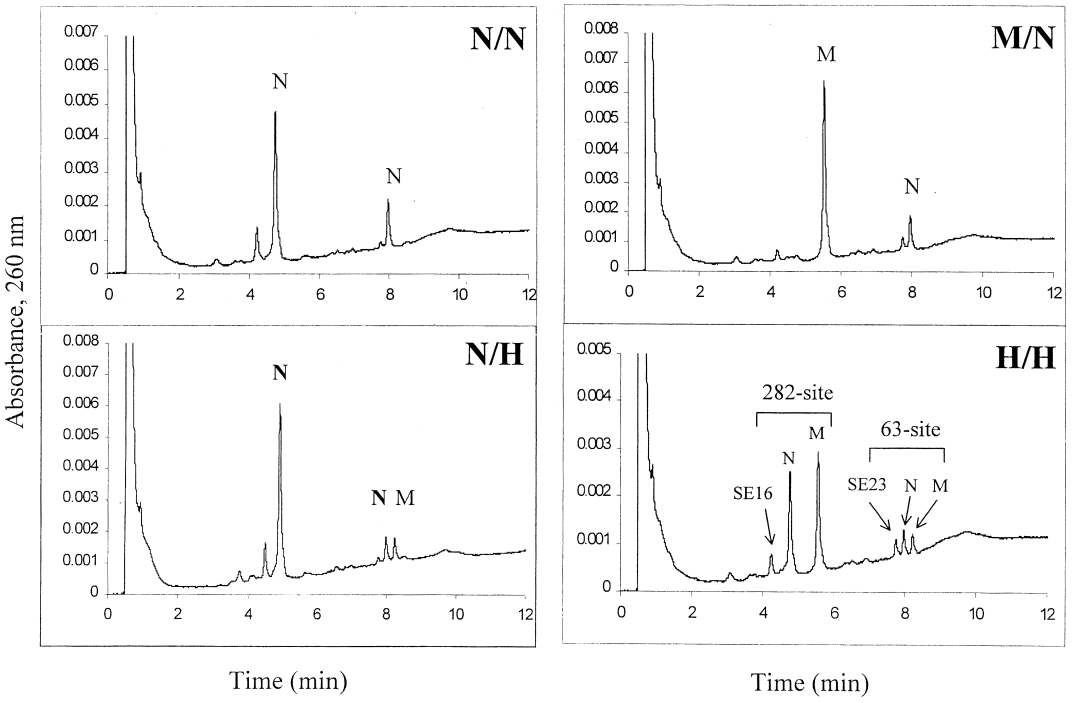

Fig. 2. HPLC chromatograms of multiplex detection of the two HH-related mutations in four genotypes, by SNE analysis.

min. To ascertain the accuracy of each method, 29 samples of known genotype were analyzed and compared with previously obtained results. In all the samples tested, both RFLP– and SNE–HPLC analysis determined the correct genotype.

We have shown that HPLC can be applied to the multiplex detection of the two common mutations associated with HH by analyzing RFLP or SNE products. RFLP products are dsDNA and size-depend for HPLC separation [17,18] while SNE products are ssDNA and both size- and sequence-dependent for separation on HPLC [19,20]. RFLP analysis is challenging for multiplex detection of more than two sites since enzymes must distinguish between the wild type and the mutant, react under similar conditions, and the digested products need to be considerable different in sizes. In comparison, the SNE method is more versatile for most polymorphism detection. Multiplexing of more than two mutations is possible using primers of different lengths or the same length, with empirical selection of detection primers. Studies have indicated that HPLC allows ssDNA less than 100 nucleotides to be separated [20] so that there is no need to generate products of various lengths for genotype determination [12]. On the other hand, since the elution time depends on both the length and sequence, it is necessary to run a set of known-genotype samples to determine the identity of each peak, as well as to make sure the chosen primers produce distinguishable peaks. In our SNE study, SE16 and SE23 gave the most easily distinguished profiles for genotyping. The primers SE16 and SE18 were not the optimum choice because the mutant 282-site product and mutant 63-site product co-elute under the current gradient conditions. In comparison to HPLC analysis of heteroduplex formation [10], both RFLP and SNE require post PCR treatment or reaction. However, heteroduplex analysis needs two HPLC analyses, with and without the addition of wild-type samples. Furthermore, RFLP and SNE are specific for the mutations of interest, while heteroduplex may result from other minor variations within the region of the PCR amplicons. For single nucleotide polymorphism analysis we found both RFLP and SNE to be valid approaches, with SNE offering a number of advan-

tages which make it a more versatile method especially for multiplex detection of more than two mutations.

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