methods

Denaturing high-performance liquid chromatography in the detection of ABCA1 gene mutations in familial HDL deficiency[S]

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Abstract **Mutations in the ABCA1 gene are the cause of familial high density lipoprotein deficiency (FHD). Because these mutations are spread over the entire gene, their detection requires the sequencing of all 50 exons. The aim of this study was to validate denaturing high-performance liquid chromatography (DHPLC) in mutation detection as an alternative to systematic sequencing. Exons of the ABCA1 gene were amplified using primers employed for sequencing. Temperatures for DHPLC were deducted from a software and empirically defined for each amplicon. To assess DHPLC reliability, we tested 30 sequence variants found in FHD patients and controls. Combined DHPLC and sequencing was applied to the genotyping of new FHD patients. Most of the amplicons required from two to five temperature conditions to obtain partially denatured DNA over the entire amplicon length. Twenty-nine of the variants found by sequencing were detected by DHPLC (97% sensitivity). The detection of the last variant (in exon 40) required different primers and amplification conditions. DHPLC and sequencing analysis of new FHD patients revealed that all amplicons showing a heteroduplex DHPLC profile contained sequence variants. No variants were detected in amplicons with a homoduplex profile. DHPLC is a sensitive and reliable method for the detection of ABCA1 gene mutations.**—Fasano, T., L. Bocchi, L. Pisciotta, S. Bertolini, and S. Calandra. **Denaturing high-performance liquid chromatography in the detection of ABCA1 gene mutations in familial HDL deficiency.** ***J. Lipid Res.*** **2005.** 46: **817–822.**

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Mutation analysis of disease genes requires sensitive, specific, and inexpensive technologies for the detection of sequence variations (both rare pathogenic mutations and common nucleotide polymorphisms). Ideally, such technologies should be capable of fully automated high-throughput analysis of simple PCR products (that do not require post-PCR manipulations). Well-established techniques for mutation detection include relatively simple methods [such as single-strand conformation polymorphism (SSCP)] as well as more complex procedures [denaturing gradient gel electrophoresis (DGGE) or enzymatic and chemical cleavage] (1). Some of these methods, such as SSCP, have low sensitivity, whereas others, such as DGGE, are time-consuming, expensive, and difficult to apply to a large number of samples or to be automated (1).

Denaturing high-performance liquid chromatography (DHPLC) is a relatively novel technique that appears to meet the requirements specified above for large-scale screening of sequence variants. This technique is based on the detection of heteroduplexes in PCR products by ion-pair reversed-phase liquid chromatography under partial denaturing conditions within an acetonitrile gradient. Heteroduplexes and homoduplexes are the result of chance reassociation of DNA strands after a denaturation cycle in a heterozygote sample.

The presence of a sequence mismatch in heteroduplexes leads to a reduced column retention time compared with their homoduplex counterparts. The major advantages of this method are the use of automated instrumentation and the speed of analysis (2).

Numerous reports during the last few years have documented the high accuracy and excellent sensitivity of DHPLC (96–100%) in detecting mutations in more than 250 genes (3). DHPLC appears to be a reliable method specifically for the analysis of large genes known to be highly polymorphic and with a large variety of pathogenic mutations (2, 3).

The ABCA1 gene is a large gene of 50 exons located on chromosome 9 (9q31) (4). The product of this gene is a full-membrane ABC transporter of 2,261 amino acids that pro-

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motes the transfer of cholesterol and phospholipids from the cell membrane to an extracellular acceptor such as lipid-poor apolipoprotein A-I (5). This process, specifically in hepatocytes, is one of the major mechanisms of plasma HDL formation (6, 7).

Rare mutations in the ABCA1 gene in homozygous and compound heterozygous states are the cause of severe plasma HDL deficiency (analphalipoproteinemia), a recessive disorder known as Tangier disease (TD) (8). TD is a systemic disease characterized by several clinical manifestations (e.g., hepatosplenomegaly, anemia, peripheral neuropathy, etc.) and an increased predisposition to cardiovascular disease (8). In the heterozygous state, rare ABCA1 mutations represent one of the causes of a genetically heterogeneous codominant disorder known as familial high density lipoprotein deficiency (FHD; or primary hypoalphalipoproteinemia) (8–10). FHD caused by ABCA1 mutations is associated with a 40–45% reduction of plasma HDL levels and increased cardiovascular risk (11).

To date, more than 50 mutations of the ABCA1 gene have been reported in TD and FHD (12). A list of pathogenic mutations can be found at http://www.abca1-mutations.all.at (13). In addition, several common nucleotide polymorphisms in the coding region and in introns have been described (14–17).

Because rare pathogenic mutations are spread over the entire ABCA1 gene, their detection requires the systematic analysis of all exons and exon/intron boundaries of this gene. Several groups, including ours, have used complete or partial gene sequencing for mutation detection (12, 13, 18–20). Although this approach has been successful, it is time-consuming and does not allow large-scale analysis of FHD patients. The present study was designed to define the most suitable experimental conditions for extending the use of DHPLC to the analysis of the ABCA1 gene. We took advantage of all of the sequence variants we identified in this gene during the genotyping of subjects with TD and FHD.

METHODS

Selection of patients with plasma HDL deficiency

Details of patients with TD or FHD carrying known mutations of the ABCA1 gene have been previously reported by our group (18–20). Three new patients (probands T.M., C.R., and B.V.) with the clinical diagnosis of FHD were investigated during the course of this study. All of these patients had low plasma levels of HDL-cholesterol (HDL-C) (21–30 mg/dl) in the absence of overt clinical manifestations or other abnormal laboratory tests. Low plasma HDL-C levels were transmitted as a codominant trait in the kindred of proband T.M. No family members were available for study in the case of the other two patients. Three control subjects, in whom the entire ABCA1 gene had been sequenced, were randomly selected from healthy normolipidemic individuals. Fifty randomly selected individuals were recruited for the screening of the new putative pathogenic mutation (c.5689C>T) found in proband T.M.

Informed consent was obtained from all subjects investigated. The study protocol was approved by the institutional human investigation committee of each participating institution.

PCR amplification conditions for direct sequencing of the ABCA1 gene

For PCR amplification of the 50 ABCA1 exons, we used the primer pairs listed in supplementary Table I.

Genomic DNA isolated from peripheral blood cells was amplified in a 50 μl volume containing 100 ng of DNA, 0.1 mM deoxynucleoside triphosphate (dNTP), 2 mM $MgCl_2$, 75 pmol of primers, and 2 units of Expand High Fidelity Taq DNA polymerase (Roche Diagnostics GmbH, Penzberg, Germany). The PCR amplification conditions for exons 1–8, 10–15, 17–24, 26, 27, 29, 32, 33, 35, 38, and 40 were as follows: 95°C for 3 min and 29 cycles of 95°C for 40 s, 60°C for 40 s, and 68°C for 2 min. The amplification conditions for exons 9, 16, 25–28, 30, 31, 34, 36, 37, 39, and 41–50 were as follows: 95°C for 3 min and 29 cycles of 95°C for 40 s, 63°C for 40 s, and 68°C for 2 min.

PCR amplification conditions for DHPLC analysis of the ABCA1 gene

The primer pairs listed in supplementary Table I were also used to amplify the ABCA1 gene for DHPLC analysis.

Genomic DNA isolated from peripheral blood cells was amplified in a 50 μl volume containing 50–300 ng of DNA, 0.2 mM dNTP, 1.5 mM $MgCl_2$, 25 pmol of primers, and 1.75 units of Optimase™ polymerase (Transgenomic™, Cheshire, UK). PCR amplification conditions for exons 2–8, 10–15, 17–24, 26, 27, 29, 32, 33, 35, 38, and 40 were as follows: 95°C for 3 min and 29 cycles of 95°C for 40 s, 60°C for 40 s, and 72°C for 2 min. PCR amplification conditions for exons 9, 16, 25–28, 30, 31, 34, 36, 37, 39, 41–47, 49, and 50 were as follows: 95°C for 3 min and 29 cycles of 95°C for 40 s, 63°C for 40 s, and 72°C for 2 min. For the other exons, the PCR amplification conditions were as follows: *i*) 95°C for 2 min and 29 cycles of 95°C for 30 s, 61.4°C for 30 s, and 72°C for 50 s followed by an extension at 72°C for 5 min (exon 1); or *ii*) 95°C for 2 min and 29 cycles of 95°C for 30 s, 58.7°C for 30 s, and 72°C for 30 s followed by an extension at 72°C for 5 min (exon 48).

In some experiments, PCR amplification of exon 40 was conducted using a different set of primers (see supplementary Table I) and the following amplification conditions: 95°C for 2 min, 15 cycles at 95°C for 30 s, 48.9°C (−0.5°C for cycle) for 30 s, and 72°C for 20 s, and 20 cycles at 95°C for 30 s, 41.9°C for 30 s, and 72°C for 20 s followed by an extension at 72°C for 5 min.

DHPLC analysis

Each amplicon was heated at 95°C for 5 min and cooled slowly over 30 min at room temperature to promote heteroduplex formation. Between 5 and 15 μl of crude sample was loaded on a DNasep column (Transgenomic™) and subjected to DHPLC analysis using a WAVE DHPLC instrument (Transgenomic™). The reverse-phase gradient was formed by mixing buffer A (0.1 mM Triethylammonium acetate (TEAA), pH 7.0) and buffer B (0.1 mM TEAA and 25% acetonitrile, pH 7.0). Oven temperature for the optimal separation of heteroduplex molecules in each amplicon was deducted from the Transgenomic software (Navigator™ software).

In many instances, it was necessary to add other temperatures (empirically determined) to the one predicted by the software to obtain a DNA helical fraction of between 30% and 99% over the entire length of the amplicon.

To reveal sequence variants in the homozygous state (which might have been undetected in a specific DNA sample), each amplicon was mixed with an equal amount of a known homozygous wild-type amplicon. The mixed samples were then denatured at 95°C for 5 min and cooled over 30 min at room temperature before DHPLC analysis.

Sequence analysis

For each amplicon analyzed by DHPLC, DNA was reamplified with the primers listed in supplementary Table I, sequenced in both directions using a fluorescence-based method (Big Dye Terminator cycle sequencing kit; Applied Biosystems, Monza, Italy), and analyzed with an ABI PRISM 3100 DNA sequencer (Applied Biosystems). New sequence variants were confirmed by a second PCR amplification and resequencing.

Direct sequencing of the ABCA1 gene from genomic DNA was performed as specified above.

RESULTS

Selection of temperature for DHPLC analysis

The first step of our work was the identification of the most suitable experimental conditions for the DHPLC analysis of all ABCA1 amplicons. We first analyzed control DNA samples to obtain a typical wild-type elution profile for each amplicon.

The temperatures for DHPLC analysis of each ABCA1 amplicon are listed in supplementary Table II. We empirically determined the optimum temperatures of analysis to ensure that the partial DNA denaturation (30–99% helical fraction) was achieved over the entire length of the amplicon. The results of these experiments showed that the software-predicted temperature was suitable for the analysis of four amplicons only (exons 15, 22, 23, and 24); most of the amplicons required from two (e.g., exons 5, 11, 18, etc.) to five (exons 30 and 40) temperatures. Multiple temperatures were required if the amplicon had multiple melting domains.

Figure 1 shows the melting profile of exon 15, which is characterized by a single melting temperature (61.4°C). The few amplicons with this melting profile could be efficiently analyzed at the software-predicted temperature, because at this temperature the entire DNA was under partially denaturing conditions (Fig. 1B). **Figure 2** shows the complex melting profile of exon 30. At the software-deducted temperature (55.1°C), only part of the DNA (the first 90 bp of the 5′ end of the amplicon) was under partially denaturing conditions. By adopting a set of higher temperature conditions (57.1, 59.1, 61.1, and 62.1°C), we were able to obtain a partially denatured DNA over the entire length of the amplicon (Fig. 2B).

Usually, the empirically defined temperatures were higher than the ones predicted by the software. Amplicons containing exons 33, 34, 37, and 45, however, were also analyzed at a temperature lower than that deducted from the software (see supplementary Table II). Lower temperatures were required when the 5′ end of an exon was close to the 5′ end of the amplicon.

The temperatures required for the analysis of an amplicon with multiple melting domains spanned a 6–7°C range, but the maximum difference between two neighboring temperatures was ±2°C.

Identification of sequence variants by DHPLC

To ascertain whether we were able to detect sequence variants by DHPLC, we applied the experimental conditions listed in supplementary Table II to a pool of positive control samples, previously analyzed by direct sequencing. Supplementary Tables IIIA, B and IV show the nucleotide variants we had previously detected in the ABCA1 gene in patients with either TD or FHD and control subjects by direct sequencing (15 common variants in the coding region: 6 intronic variants and 9 pathogenic mutations).

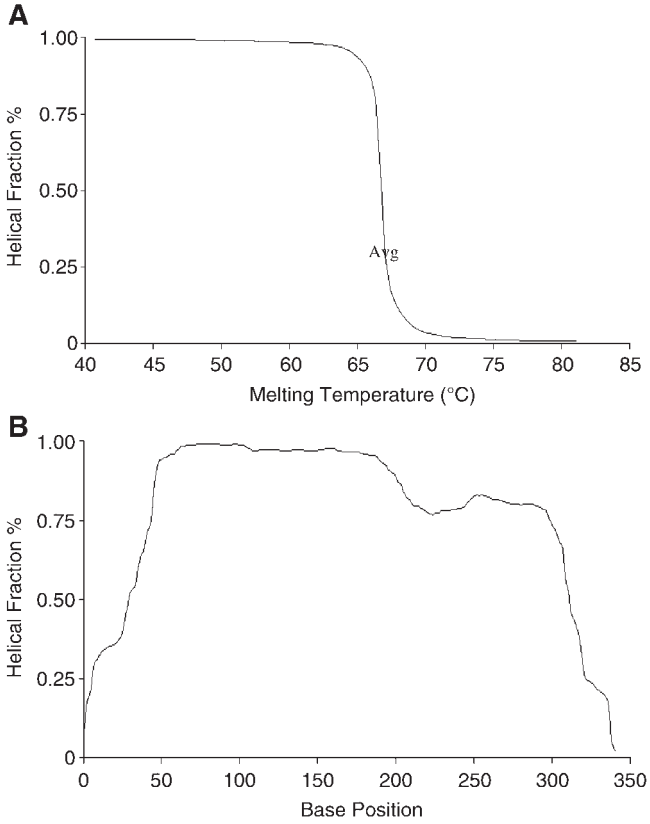

Fig. 1. A: Melting profile of the ABCA1 gene amplicon containing exon 15. Avg, average. B: Profile of the percentage of DNA helical fractions over the entire length of the same amplicon at the temperature (61.4°C) deducted from the Transgenomic™ software (see supplementary Table II).

Only the amplicons harboring known sequence variants (with the exception of that corresponding to exon 40) showed an elution profile suggestive of the presence of a heteroduplex. Therefore, the sensitivity of DHPLC for ABCA1 gene amplicons obtained with the primers originally adopted for direct sequencing was close to 97%.

The amplicon containing exon 40 showed a complex elution profile, with multiple peaks in both the wild-type and mutant PCR products, that was difficult to interpret and that did not allow us to detect the sequence variant we expected to be present in exon 40 (see supplementary Table IV). We thought that this unsatisfactory result was dependent on the presence of a long poly-T sequence located upstream from the 5′ end of exon 40. To overcome this problem, we used a different set of primers that excluded the poly-T sequence from the amplicon (see supplementary Table I) and modified the amplification conditions (see Methods). In this way, we were able to obtain a clear elution profile in the wild-type amplicon (homodu-

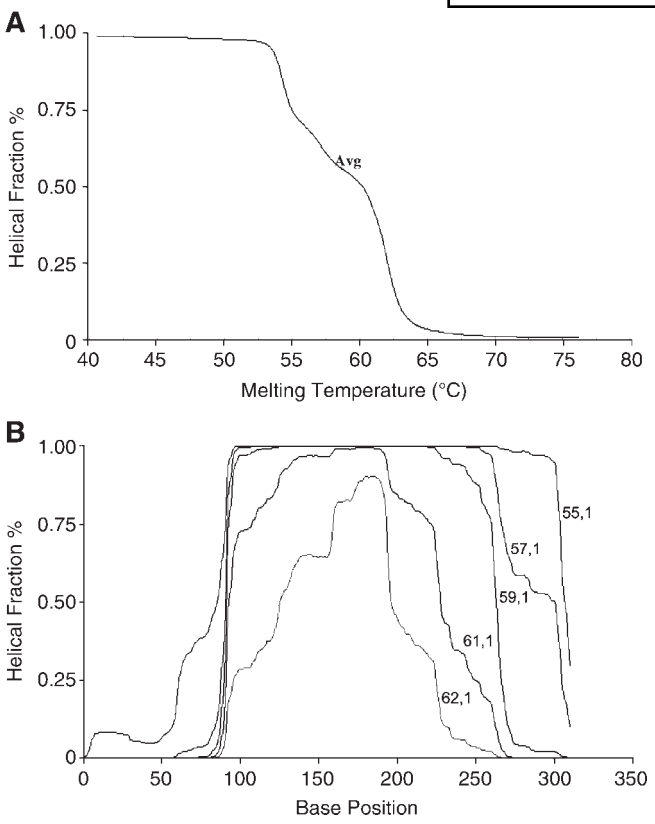

Fig. 2. A: Melting profile of the ABCA1 gene amplicon containing exon 30. Avg, average. B: Profile of the percentage of DNA helical fractions over the entire length of the same amplicon at the temperature (55.1°C) deducted from the Transgenomic™ software and at the four empirically defined temperatures (57.1, 59.1, 61.1, and 62.1°C) (see supplementary Table II).

plex) and a clear heteroduplex profile in the amplicon harboring the previously detected mutation in exon 40.

Specificity of the DHPLC elution profile

We then tried to assess whether it was possible to assign a specific heteroduplex elution profile to a sequence variant found in an amplicon. More specifically, we asked whether there were common variants with specific DHPLC elution profiles that allowed their unequivocal identification before sequencing. If that had been the case, less sequencing work would have been required for mutation identification. In the case of exons 3, 7, 32, and 35, the observation of a highly reproducible elution profile provided a strong indication of the presence of a common sequence variant (see supplementary Fig. I). This assumption could not be applied to other amplicons, as in the case of exon 9.

This amplicon can harbor two common polymorphisms (c.936C>T and c.948G>A; see supplementary Table IIIA), two rare pathogenic mutations (c.844C>T and c.850G>A; see supplementary Table IV), and one insertion in intron 8 (c.814-14 insA; see supplementary Table IIIB). As a matter of fact, the elution profile we observed did not allow differentiation among these variants, regardless of the temperature conditions selected for the analysis. In the case of the coexistence of two exonic polymorphisms (c.936C>T and c.948G>A; see supplementary Table IIIA), in the same amplicon the elution profile observed at 61°C was more complex with respect to that observed in the amplicons containing either of these variants (thus providing some clue for the coexistence of the two polymorphisms) (data not shown).

Neither of the two rare pathogenic mutations (c.844C>T and c.850G>A; see supplementary Table IV) that had been found in exon 9 of two patients with HDL deficiency showed a specific elution profile that could discriminate them from either of the two common polymorphisms that may be present in the corresponding exon.

Application of DHPLC to the detection of ABCA1 gene mutations in patients with the clinical diagnosis of FHD

We then applied DHPLC analysis to the study of the ABCA1 gene in three subjects with the clinical diagnosis of FHD. In proband T.M., 15 DNA amplicons showed a heteroduplex elution profile and 35 had a homoduplex profile (even after mixing with corresponding known homozygous wild-type amplicons). The nucleotide sequences confirmed the presence of sequence variants (in the heterozygous or homozygous state) in all of the "suspected" 15 amplicons, as indicated in supplementary Table V. Proband T.M. was found to carry most of the nucleotide variants listed in our database (see supplementary Table IIIA, B) and two additional variants in exon 25 and intron 0, respectively. In addition, he was found to be heterozygous for a novel nucleotide change in exon 42 (c.5689C>T) causing a nonsynonymous amino acid substitution (R1897W). This variant was found in the mother and the brother of this patient, who had plasma HDL levels in the lower range of the normal values. The sequences of the other 35 amplicons did not reveal nucleotide variants in either the heterozygous or the homozygous state.

In probands C.R. and B.V., all amplicons (20 in all) with a heteroduplex elution profile were found to harbor sequence variants (see supplementary Tables VI, VII). None of the other amplicons with a homoduplex DHPLC profile was found to contain sequence variants.

All coding sequence variants found in probands C.R. and B.V. have been previously reported as common polymorphisms. In probands C.R. and B.V., we found three new intronic variants (c.6069+27T>C, c.6401+13A>G, and c.6401+86A>C) that we assumed to have no pathogenic effect, being located outside the canonical splice sites. The automated splice site analysis (https://splice.cmh.edu/) (21) used to ascertain whether these intronic variants reduced the information content of the splice sites of the corresponding intron did not reveal changes in the natural splicing sites.

Use of DHPLC for mutation screening

To ascertain whether the novel nucleotide substitution in exon 42 (c.5689C>T, R1897W) found in proband T.M. was a previously undetected common polymorphism, we used DHPLC to screen 50 randomly selected control subjects (see supplementary Fig. II). None of them was found to carry this mutation. This result was confirmed by sequencing.

DISCUSSION

In this study, we applied DHPLC analysis to the detection of sequence variants in the ABCA1 gene, a large human gene with several common sequence variants and many rare pathogenic mutations.

To compare the results of our previous sequencing work with those obtained with DHPLC, we used the same primers previously used for sequencing (with only one exception in the case of exon 40, as specified below). We reasoned that this choice of primers would lead to a more profitable integration between DHPLC and sequencing in mutation detection and identification. As a first step in DHPLC validation, we defined the temperatures at which in each amplicon DNA was under partial denaturing conditions suitable for DHPLC analysis. We found that under our experimental conditions, DHPLC analysis required two or more temperatures in most cases. This is likely attributable to the size of the amplicons and the complexity of their melting profiles (Fig. 2). We are aware that the analysis of an amplicon at different temperatures is time-consuming with respect to that performed at the single temperature deducted from the DHPLC software. If we assume that a single DHPLC analysis requires 7–8 min, the time required for the analysis of the same amplicon at five different temperatures plus the time needed for the oven to reach the new temperature (~2–3 min) could be as long as 60–70 min. However, we demonstrated that all of the temperatures selected for each amplicon are needed to screen the amplicon in its entire length.

The choice of the appropriate temperature conditions allowed us to detect 29 of 30 known sequence variants, confirming that under our experimental conditions DHPLC has a sensitivity of 97%, similar to that reported for other genes (3).

We failed to obtain a clear elution profile from the analysis of the amplicon containing exon 40 using our original set of primers (see supplementary Table I). On the assumption that this result was attributable to the presence of a long poly-T sequence at the 5′ end of the amplicon, we amplified the DNA fragment using a new set of primers and different PCR conditions (see Methods). The shorter amplicon we obtained was amenable to DHPLC analysis; under these new conditions, we were able to confirm the presence of a rare pathogenic mutation in exon 40 (c.5398A>C, R1800H).

DHPLC analysis applied to ABCA1 genotyping of three new patients with the clinical diagnosis of FHD revealed that 20–30% of ABCA1 amplicons showed a heteroduplex profile. Sequencing demonstrated that all of them contained known or unknown sequence variants. On the other hand, none of the amplicons with a homoduplex elution profile was found to harbor sequence variants, indicating that no false-positive or false-negative results are expected in DHPLC analysis of the ABCA1 gene under our experimental conditions.

The observation that in patients with the clinical diagnosis of FHD ~30% of the amplicons show a heteroduplex profile in DHPLC raises the question of the great sequencing work required to identify the type of sequence variants to detect possibly pathogenic mutations. Given the fact that accurate genotyping imposes the sequencing of all "heteroduplex positive amplicons," a laboratory staff experienced in the analysis of the ABCA1 gene may suggest the sequencing first of those amplicons that are known not to harbor common sequence variants. In this way, rare and possibly pathogenic mutations may be identified more quickly.

Among the novel sequence variants found in the new patients with FHD, the one found in exon 42 of proband T.M. likely represents a pathogenic mutation for several reasons: *i*) it cosegregated with low plasma HDL-C in the family; *ii*) it was not found in 50 randomly selected control subjects; *iii*) it involved an amino acid residue (R1897) that is conserved in mice; and *iv*) it is predicted to be "possibly damaging" in terms of protein function and structure according to the prediction [Position Specific Independent Counts (PSIC) score difference of 1.983] generated by the computer program (PolyPhen; http://tux.embl.heidelberg.de/ramensky/polyphen.cgi).

In conclusion, we have demonstrated the feasibility of DHPLC analysis as a new sensitive and rapid method for the analysis of the ABCA1 gene in patients with the clinical diagnosis of TD or FHD. DHPLC may be cost-effective in the diagnosis of these disorders compared with other analytical methods (SSCP and DGGE) and a valuable alternative to the direct sequencing of all 50 amplicons of the ABCA1 gene.

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