Cascade Screening for Familial Hypercholesterolemia: PCR Methods with Melting-Curve Genotyping for the Targeted Molecular Detection of Apolipoprotein B and LDL Receptor Gene Mutations to Identify Affected Relatives

Sarojini Pandey,1 Michaela Leider,2 Mike Khan,3 and Dimitris K. Grammatopoulos1,4*

Background: A key objective of the UK National Institute for Health and Care Excellence (NICE) pathway for diagnosis of familial hypercholesterolemia (FH) is the identification of affected relatives of index cases through cascade screening. At present, there is no systematic appraisal of available methodological options to identify the appropriate diagnostic testing protocol that would allow cost-effective cascade genetic screening. The majority of FH-causing mutations identified in the LDL receptor (LDLR) or apolipoprotein B (APOB) genes are single-nucleotide changes. This pattern of mutations suggests that PCR methods using melting curve–based genotyping might offer a convenient methodological approach for screening relatives.

Methods: We developed and validated one-tube PCR methods for the mutations APOB c.10580G>A (p.Arg3527Gln), LDLR c.1474G>A (p.Asp492Asn), and c.2054C>T (p.Pro685Leu) and 3 novel LDLR mutations identified in the Coventry and Warwickshire population: LDLR c.1567G>C (p.Val523Leu), c.487dupC (p.Gln163Profs17), and c.647G>C (p.Cys216Ser).

Results: These methods successfully amplified target sequence from genomic DNA extracted from either peripheral blood or saliva. They also demonstrated acceptable analytical performance characteristics (specificity of amplification, repeatability, and reproducibility) over a wide range of DNA concentrations and purity. This approach was used for cascade testing of relatives of index FH cases with confirmed mutations and identified family members with high plasma LDL cholesterol as heterozygous for disruptive alleles.

Conclusions: Our study generates proof-of-concept evidence of methods suitable for detecting single nucleotide substitutions and insertions that can deliver reliable, easy, low-cost, and rapid family screening of FH patients and can be adopted by nonspecialist molecular diagnostic laboratories.

1Department of Clinical Biochemistry, University Hospital Coventry and Warwickshire, Coventry, UK; 2TIB MOLBIOL Syntheselabor GmbH, Berlin, Germany; 3Department of Endocrinology and Diabetes, University Hospital Coventry and Warwickshire, Coventry, UK; 4Division of Translational and Systems Medicine, Warwick Medical School, Coventry, UK

*Address correspondence to this author at: Warwick Medical School, University Hospital Campus, Clifford Bridge Road, Walsgrave, Coventry CV2 2DX, UK. Fax 44-2476-574637; e-mail d.grammatopoulos@warwick.ac.uk.

DOI: 10.1373/jalm.2016.020610

© 2016 American Association for Clinical Chemistry

Nonstandard abbreviations: FH, familial hypercholesterolemia; LDL-C, LDL cholesterol; NICE, National Institute for Health and Care Excellence; Tm, melting temperature.

Human genes: LDLR, LDL receptor; APOB, apolipoprotein B; PCSK9, proprotein convertase subtilisin/kexin type 9; LDLRAP1, LDL receptor adaptor protein 1.
Patients with familial hypercholesterolemia (FH) will benefit from the information presented here. It is estimated there are 34 million FH cases worldwide with <1% having been identified in most countries. Evidence presented on the development of novel PCR-based approaches will allow better and more cost-effective delivery of the cascade testing of the FH pathway. Knowledge in the field of molecular genetics relevant to lipid disorders will be advanced by the information presented.

Familial hypercholesterolemia (FH)\(^5\) (OMIM #606945) is an autosomal-dominant disorder associated with abnormally high serum concentrations of LDL cholesterol (LDL-C) \(^1\). Affected individuals have increased risk of premature coronary heart disease and death \(^2\). FH is one of the most common inherited disorders, with an estimated worldwide prevalence of 1 in 500 (0.2%), although the frequency is considerably higher in some populations, and recent reports increase the estimate of prevalence to 1/200 \(^3\), thus raising a strong possibility of disease underdiagnosis.

More than 80% of FH patients have mutations in the LDL receptor \(\text{LDLR}\)\(^6\) which leads to defective uptake or processing of LDL particles \(^4\). Disease-causing mutations have also been identified in apolipoprotein B \(\text{APOB}\) and in protein convertase subtilisin/kexin type 9 \(\text{PCSK9}\) genes; these comprise around 5% and 2% of all UK FH mutations, respectively \(^5\). Mutations have also been identified in the LDL receptor adaptor protein 1 \(\text{LDLRAP1}\) gene, seen in a recessive form of FH \(^6\). Despite their greatly increased risk of coronary heart disease, most individuals with FH remain undiagnosed, untreated, or inadequately treated. The majority are heterozygotes and a smaller number (approximately 1/200 000) are compound heterozygotes, while homozygotes are extremely rare (approximately 1/1 million). Heterozygous FH patients exhibit a cumulative risk for coronary heart disease >50% in men by age 50 years and at least 30% in women by age 60 years \(^7\). Early detection of the disease and treatment has been shown to reduce morbidity and mortality \(^8\). In the UK, it is estimated that the identification and optimal treatment of all FH cases could save the National Health Service £380 million ($570 million) over a 55-year period, or £6.9 million ($10.3 million)/year \(^9\); in the European Union, the healthcare cost savings would be around €86 million ($129 million) per year \(^10\). However, despite efforts to improve the identification and management of FH patients, few countries have established large-scale programs to systematically identify index cases and screen their relatives. It is estimated that <25% of FH cases in the UK and <1% in the US have their molecular defect identified \(^11, 12\).

In 2008, the National Institute for Health and Clinical Excellence (NICE) in the United Kingdom developed guidelines on FH management strongly recommending identification of causal mutations in suspected cases of FH phenotype and cascade screening using a combination of genetic testing and LDL-C concentration measurement to identify affected relatives of those index individuals with a clinical diagnosis of FH, including the first-, second-, and third-degree biological relatives \(^13\). Genetic testing of affected individuals and screening of relatives is considered the most cost-effective strategy for detecting cases of FH \(^14\) and for distinguishing monogenic FH from sporadic or polygenic hypercholesterolemia \(^15\).

Over 1400 different mutations are listed in \(\text{LDLR}\) gene database of University College London.
Mutations are distributed throughout the whole gene sequence with no apparent hotspot and large rearrangements that comprise around 11% of all LDLR mutations (4). Moreover, it is becoming evident that, in the UK, there is considerable regional variation in the profile of FH-associated mutations that might be relevant to population ethnic background and migration patterns; for example, a recent pilot study in a West Midlands cohort showed increased frequency of the APOB 10580G>A mutation among FH patients (17).

The spectrum of FH-associated mutations has made screening challenging, especially for frontline clinical diagnostic laboratories that offer molecular testing alongside biochemical assessment of FH patients. NICE recommends comprehensive genetic analysis of the LDLR, plus analysis for the common APOB and PCSK9 mutations, as the most effective test in terms of sensitivity and quality-adjusted life years (18). NICE also recommends targeted gene resequencing for investigation of biological relatives of index cases, a method that usually involves isolation of genomic regions of interest after generation of sequencer-ready DNA fragment libraries enabling systematic detection of DNA variants of interest.

These methodologies are quite complex and require specialist expertise and dedicated infrastructure not readily available to all frontline clinical diagnostics services that are expected to deliver the FH testing program. Instead, selective testing using simple PCR-based formats might be a suitable alternative especially to deliver the second-line cascade testing and identify affected relatives of index cases. Here, we describe the development and validation of low-cost PCR methods using melting-curve based genotyping with fluorescence-labeled hybridization probes for detection of number of APOB and LDLR mutations. We applied these methods for cascade testing and screening first-degree relatives of patients diagnosed with FH clinical phenotype and were identified as heterozygous for either the APOB 10580G>A or LDLR 2054C>T mutations.

MATERIALS AND METHODS

Patients

Index cases. Archived genomic DNA from FH patients with confirmed mutations was used for development of the PCR-based mutation detection methods.

Family studies. Clinical details of the families investigated are shown in the Supplementary Methods in the Data Supplement that accompanies the online version of this article at http://www.jalm.org/content/vol1/issue2.

Biological samples (blood or saliva) were obtained and processed for DNA extraction. All procedures were in accordance with the Helsinki Declaration. The study was approved by the Arden Tissue Bank Ethics Committee (12/SC/0526). Supplementary Table S1 in the online Data Supplement shows family member demographics and lipid profile.

DNA extraction and PCR genotyping

Genomic DNA was extracted from saliva or EDTA-containing blood samples using a QIAamp DNA Blood Mini Kit (Qiagen), and DNA concentration was quantified by ND-1000 spectrophotometer (NanoDrop, Thermo Scientific). For mutation genotyping, LightMix® and LightSNP® assays developed by TIB MOLBIOL were used (see Supplementary Methods in the online Data Supplement). An example of the combination of primers used for amplification of a DNA fragment containing the LDLR c.1474G>A mutation and confirmation by Sanger sequencing is shown in Supplementary Fig. 1.

Analysis was performed in the LightCycler 2.0 instrument (Roche Molecular Diagnostics). The PCR
protocols are described in detail in the Supplementary Methods in the online Data Supplement.

RESULTS

Analytical performance of the genotyping methods

Detection of each mutation in genomic DNA was based on melting curve–based genotyping with fluorescence-labeled probes. For example, the presence of 10580G>A mutation was investigated in a 77-bp amplicon of APOB; the G>A substitution led to a melting temperature (Tm) shift of 7 °C ± 1.5; heterozygotes showed two distinct melting peaks, at 62 °C ± 2.5 and 55 °C ± 2.5, representing mutant and wild-type alleles, respectively (Fig. 1A). Similarly, the presence of a C>T mutation led to a Tm shift of 8.2 °C ± 1.5; heterozygotes show two distinct melting peaks, at 64 °C ± 2.5 and 55.5 °C ± 2.5, for the mutant and wild-type alleles, respectively (Fig. 1B). The Tm shift for each of the 6 mutations tested is shown in Table 1.

DNA extracted from index cases containing the relevant mutation was used as a positive control to confirm assay accuracy. The analytical specificity of the PCR methods was confirmed by parallel capillary (Sanger) sequencing of the amplified DNA sequence, and all PCR methods showed 100% concordance.

Repeatability/reproducibility studies where 5 replicates of the same DNA sample were genotyped or different batches of DNAs of heterogeneous genotypes were analyzed 5 times showed no intra-patient or between-batch variation. All LightCycler assays consistently identified the genotype correctly, confirming their analytical reliability and suitability for routine use (data not shown).

We also determined the PCR method performance across a range of DNA sample concentrations and purity. Six genomic DNA samples were...
serially diluted to provide a range of concentrations between 1.65 and 92.9 ng/μL and DNA purity (ratio of absorbance 260/280 0.77–2.56). Across this range, the LightCycler PCR methods generated melting curves with robustly discernible different melting peaks that could be detected above the background (within-run and between runs) between wild-type and heterozygous DNA (Fig. 2). The APOB c.10580G>A genotyping method was also tested in a saliva DNA sample with a concentration of 31.6 ng/μL and an A260/280 ratio of 1.75. Fig. 3 shows successful amplification and melting curve analysis of the 77-bp DNA fragment that did not contain the G>A single nucleotide substitution.

We also tested performance and possible method interference from other mutations by mixing the equivalent amount of DNA from patients with different LDLR mutations. We focused on the LDLR c.2054C>T, which exhibits increased prevalence frequency in our local population (17). The LightSNiP® rs28942084 LDLR [P685L] PCR method was tested in a DNA mix containing both c.2054C>T and c.1474G>A mutations. As confirmed by Sanger sequencing, the method robustly amplified the expected nucleotide sequence within exon 14, and the DNA amplicon with a Tm of 64 °C ± 2.5 correctly identified the C>T mutation confirming PCR specificity (data not shown).

### Table 1. The peak $T_m$ of wild-type and mutant alleles and the melting temperature shift for each of the 6 mutations tested.

<table>
<thead>
<tr>
<th>Gene mutation</th>
<th>Wild-type $T_m$</th>
<th>Mutant $T_m$</th>
<th>Melting temperature, Δ</th>
</tr>
</thead>
<tbody>
<tr>
<td>APOB c.10580G&gt;A</td>
<td>55 °C ± 2.5</td>
<td>62 °C ± 2.5</td>
<td>7 °C ± 1.5</td>
</tr>
<tr>
<td>LDLR c.1474G&gt;A</td>
<td>55.84 °C ± 2.5</td>
<td>62.83 °C ± 2.5</td>
<td>7 °C ± 2.5</td>
</tr>
<tr>
<td>LDLR c.2054C&gt;T</td>
<td>55.64 °C ± 2.5</td>
<td>63.8 °C ± 2.5</td>
<td>8.2 °C ± 1.5</td>
</tr>
<tr>
<td>LDLR c.1567G&gt;C</td>
<td>57.77 °C ± 2.5</td>
<td>63.80 °C ± 2.5</td>
<td>6 °C ± 1.5</td>
</tr>
<tr>
<td>LDLR c.487dupC</td>
<td>58.49 °C ± 2.5</td>
<td>63.7 °C ± 2.5</td>
<td>5.2 °C ± 1.5</td>
</tr>
<tr>
<td>LDLR c.647G&gt;C</td>
<td>58.2 °C ± 2.5</td>
<td>65.23 °C ± 2.5</td>
<td>7 °C ± 1.5</td>
</tr>
</tbody>
</table>

**Fig. 2. Results of a dilution series of a DNA sample heterozygous for the LDLR c.1474G>A mutation.**

The DNA sample neat concentration was 92.9 ng/μL and was diluted 1:5, 1:10, and 1:20 in distilled water. PCR followed by melting curve–based genotyping shows that across all samples, the wild-type allele appears as a melting peak at 55.84 °C ± 2.5 and the mutant allele appears as a melting peak at 62.83 °C ± 2.5.
Family studies

The LDLR c.2054C>T PCR method was used to genotype three first-degree relatives (two sisters and one daughter) of a 51-year-old FH female patient. The mutation was not detected in either sister; however, the 21-year-old daughter of the index case, who at presentation had an abnormal phenotype with Tchol of 7.5 mmol/l (290 mg/dL) and an LDL-C of 5.7 mmol/l (220.42 mg/dL) tested positive for the mutation (Fig. 4A). In a different family study of a 63-year-old FH female patient, both the 34-year-old son who presented with grossly increased Tchol of 9.2 mmol/l (355.76 mg/dL) and an LDL-C of 7.2 mmol/l (278.42 mg/dL) and 11-year-old granddaughter who had Tchol of 5.5 mmol/l (212.68 mg/dL) and an LDL-C of 4.0 mmol/l (154.67 mg/dL) were found positive for the LDLR c.2054C>T mutation (Fig. 4B).

The APOB c.10580G>A mutation status was investigated in 4 children of a 35-year-old FH patient. Genomic DNA was extracted from either peripheral blood or saliva samples. Lipid levels were not measured in this family. The mutation was not detected in 3 out of 4 children; the 14-year-old son...
of the index case was the only one found positive for the mutation (Fig. 5A). Similar genetic investigations were carried out in 2 daughters of a 46-year-old FH male patient. The mutation was absent in 1 sister, with Tchol of 4.7 mmol/l (181.74 mg/dL) and an LDL-C of 2.6 mmol/l (100.54 mg/dL). In contrast, the other sister presented with an abnormal phenotype with Tchol of 7.3 mmol/l (282.29 mg/dL) and an LDL-C of 5.0 mmol/l (193.35 mg/dL), supporting the diagnosis of definite FH according to the Simon Broome Criteria, and was positive for the \( \text{APOB} \) c.10580G>A mutation (Fig. 5B).

**DISCUSSION**

Implementation of the NICE pathway for diagnosis of FH requires biochemical assessment of FH patients and, in parallel, diagnostic approaches suitable for identifying the disease-causing mutation followed by appropriate testing protocols to identify affected relatives of index cases. The wide spectrum of FH-associated mutations identified in the \( \text{LDLR} \) gene, which harbors the majority of disease-causing mutations, requires molecular methods suitable for comprehensive scanning of the nucleotide sequence of the candidate genes (19, 20). Next-generation sequencing as a comprehensive genetic analysis method has demonstrated high levels of specificity and sensitivity (21–23). However, a significant drawback is the recognition that sequencing 4 genes is insufficient, and approximately 15% of people with FH do not have a mutation in \( \text{LDLR} \), \( \text{APOB} \), or \( \text{PCSK9} \) genes (though estimates range from 12% to 48%) (24).

These limitations coupled with the cost of genetic methods (over £200 ($300) per comprehensive genetic analysis and over £70 ($105) per targeted resequencing for cascade testing) limits the appeal for investment by clinical commissioners in the UK healthcare system that operates under enormous cost-saving pressures. Therefore, at present, the aim of the Department of Health’s 2013 cardiovascular disease outcomes strategy to improve the current diagnosis rate from 15% to 50% of the estimated FH cases in the UK seems overoptimistic.

Once a mutation has been identified in an index case, testing protocols to screen relatives presents little diagnostic challenge. Although NICE recommends targeted gene resequencing, other approaches might offer easier and cheaper alternative methodologies, especially since the majority of the disease-causing \( \text{LDLR} \) DNA variations are single nucleotide substitutions (76.6%, 1076/1404). Indeed, previous studies resulted in
the development of PCR-based allele-specific amplification method for the rapid detection of the LDLR C. 1646G>A G528D mutation (also known as FH-Palermo-1) (25). In this study, we report development of simple PCR-based methods designed to detect specific LDLR and APOB mutations. These PCR methods enable simultaneous amplification of both mutant and wild-type alleles and efficient and rapid discrimination between them by melting curve–based genotyping. We decided to explore this widely used analytical approach (26) that offers major advantages because of the simple format and minimal developmental time required; indeed, many LDLR mutation assays are already commercially available, and setting up a new assay for a novel mutation is generally not a challenging task. The robustness and performance characteristics of these methods was not affected by the type of nucleotide substitution that was investigated—that is, G>A, C>T, G>C (substitutions), or a single nucleotide insertion. We targeted nucleotide substitutions due to transitions that involve interchanges of either two-ring purines (G>A) or one-ring pyrimidines (C>T) as well as transversions that interchange purine for pyrimidine bases (G>C) and involve exchange of one-ring and two-ring structures. The LDLR c.487dupC frameshift mutation identified in our local population involves insertion of a cytosine (C) in a run of 5 consecutive C, a pattern seen in half of the insertion cases in LDLR, where the single nucleotide insertion occurs within runs of 2–7 identical bases (27). All methods demonstrated excellent analytical performance, even when the DNA template used had less than optimal quality and quantity. These methods offer the potential to diagnostic laboratories to screen relatives for known mutations with standard PCR protocols and are suitable for laboratories with a modest budget or where sophisticated equipment or specialist expertise is not available. The cost of each PCR method is estimated below £20 ($30), which is almost a 4-fold cost-saving from targeted sequencing approaches. Moreover, the fast turnaround time (approximately 2 h) offers a significant advantage for clinical service delivery and development of one-stop clinical appointments.

These methods were used for cascade screening to investigate relatives of FH patients identified with APOB c.10580G>A (p.Arg3527Gln) and LDLR c.2054C>T (p.Pro685Leu) mutations. The R3527Q mutation is one of the disease’s causative mutations, and its origin is placed 6000–7000 years ago (28). It is the most common mutation in APOB that occurs in exon 26, with the majority of affected patients being of Central European descent and an estimated frequency of 1/1000 (28, 29).

In conclusion, we demonstrate that LightCycler-based PCR methods targeting specific mutations offer a robust diagnostic solution for determining the mutations association with FH in family studies and have the potential to deliver the second line of investigations of the FH cascade testing pathway. This relatively simple method offers the potential for an easier, cost-effective strategy that is suitable for minimally equipped frontline clinical laboratories that do not have access to specialist equipment and scientific expertise. The fast turnaround time (approximately 2 h) of the method offers a significant advantage and could provide a faster service and support delivery models such as a “one-stop” lipid clinic. In this model, results from the molecular tests could be available to the clinical team within a few hours to fast-track clinical decision-making and choice of treatment as well as patient convenience, thus offering additional financial savings to the healthcare provider.
Author Contributions: All authors confirmed they have contributed to the intellectual content of this paper and have met the following 4 requirements: (a) significant contributions to the conception and design, acquisition of data, or analysis and interpretation of data; (b) drafting or revising the article for intellectual content; (c) final approval of the published article; and (d) agreement to be accountable for all aspects of the article thus ensuring that questions related to the accuracy or integrity of any part of the article are appropriately investigated and resolved.

Authors’ Disclosures or Potential Conflicts of Interest: Upon manuscript submission, all authors completed the author disclosure form. Employment or Leadership: None declared. Consultant or Advisory Role: None declared. Research Funding: D.K. Grammatopoulos, UHWC National Health Service Trust Research Development and Innovation (RDI) Award. Expert Testimony: None declared. Patents: None declared.

Role of Sponsor: The funding organizations played no role in the design of study, choice of enrolled patients, review and interpretation of data, or preparation or approval of manuscript.

REFERENCES


