Identification of deletions and duplications in the low density lipoprotein receptor gene by MLPA

Øystein L. Holla, Christél Teie, Knut Erik Berge, Trond P. Leren*

Medical Genetics Laboratory, Department of Medical Genetics, Rikshospitalet, N-0027 Oslo, Norway

Received 6 December 2004; received in revised form 14 January 2005; accepted 17 January 2005

Abstract

Background: Familial hypercholesterolemia (FH) is caused by mutations in the low density lipoprotein (LDL) receptor gene. In this study we have compared multiplex ligation-dependent probe amplification (MLPA) and long-range PCR to detect large deletions/duplications in the LDL receptor gene.

Method: DNA from 431 unrelated FH patients without mutations in the LDL receptor gene detectable by DNA sequencing and who had total serum cholesterol levels above 10.0 mmol/l, was subjected to analyses by MLPA and by five long-range PCRs.

Result: Eleven deletions and two duplications were detected by MLPA. Six of the deletions and one of the duplications were also detected by long-range PCR. A total of 44 of the 431 (10.2%) FH patients possessed a deletion or a duplication.

Conclusion: MLPA has a higher sensitivity than five long-range PCRs to detect large deletions/duplications in the LDL receptor gene. Even though the direct cost of MLPA is twice that of five long-range PCRs, it has replaced long-range PCR for routine diagnostics in our laboratory because of the higher sensitivity and the 30–50% reduction in hands-on time.

© 2005 Elsevier B.V. All rights reserved.

Keywords: Familial hypercholesterolemia; LDL receptor; Deletion; Duplication; MLPA; Mutation

1. Introduction

Familial hypercholesterolemia (FH) is an autosomal dominant disorder of lipid metabolism caused by mutations in the gene encoding the low density lipoprotein (LDL) receptor [1]. As a result of disruption of the normal cell surface LDL receptors, patients with FH experience increased levels of both LDL cholesterol and total serum cholesterol from birth. Heterozygotes have total serum cholesterol levels ranging from 7 to 15 mmol/l, whereas homozygotes have levels ranging from 20 to 25 mmol/l [1]. Because a high level of total serum cholesterol is a major risk factor for coronary heart disease (CHD), patients with FH have a significantly increased risk of premature CHD [1–3].

Abbreviations: CHD, coronary heart disease; FH, familial hypercholesterolemia; LDL, low density lipoprotein; MAPH, multiplex amplifiable probe hybridization; MLPA, multiplex ligation-dependent probe amplification; PCR, polymerase chain reaction.

* Corresponding author. Tel.: +47 23075552; fax: +47 23075561.
E-mail address: trond.leren@rikshospitalet.no (T.P. Leren).
FH is one of the most common genetic disorders with a prevalence of heterozygotes of about 1/500 in most western countries [4,5]. On a world-wide basis, it has been estimated that more than 10 million people have FH, of which as many as 200,000 die of premature CHD each year [6]. However, in contrast to most genetic diseases, efficient therapy is available for FH in the form of lifestyle changes and lipid-lowering drugs.

FH can be diagnosed either by clinical criteria based upon lipid levels, family history and presence of xanthomas [1,3,7] or by identification of a mutation in the LDL receptor gene by molecular genetic methods [1,8,9]. The LDL receptor gene spans 45 kb on chromosome 19 and consists of 18 exons and 17 introns [10]. The 5175 bp mRNA, of which only 2580 bp is translated, encodes the 860 amino acid receptor protein [11]. Several PCR-based methods can be used to detect point mutations and small deletions/duplications within exons [12–20], and more than 750 different mutations have been identified in the LDL receptor gene (www.ucl.ac.uk/fh).

Southern blot analysis was the original method to detect large deletions/duplications [21]. However, this is a laborious and time-consuming method that typically makes use of radioactivity and also requires substantial amounts of DNA. Moreover, an abnormal band pattern obtained by Southern blot analysis may sometimes be difficult to interpret in terms of identifying the deletion/duplication break points. For these reasons, Southern blot analysis has more or less been replaced by the more convenient long-range PCR method where fragments up to 20 kb may be amplified [22,23]. However, in order to achieve a high sensitivity of long-range PCR to study genes of more than 10–20 kb in size, several overlapping amplicons need to be analyzed. Even then the sensitivity of detecting duplications in heterozygous patients is low, because amplicons containing duplications are poorly amplified when compared to the shorter wild-type amplicons.

Two new PCR-based methods have recently been developed to detect large deletions/duplications. These are multiplex amplifiable probe hybridization (MAPH) [24] and multiplex ligation-dependent probe amplification (MLPA) [25]. They both have the potential to be sensitive methods to detect large deletions and duplications. In this study we have compared MLPA and five long-range PCRs to detect large deletions/duplications in the LDL receptor gene in patients with a clinical diagnosis of FH and total serum cholesterol levels above 10 mmol/l. None of the patients possessed mutations in the LDL receptor gene detectable by DNA sequencing of the promoter region and of the individual exons with flanking intron sequences.

2. Materials and methods

2.1. Patients

Our laboratory is the only laboratory in Norway performing genetic testing for FH. Of the approximately 4800 unrelated patients who have been referred to our laboratory for genetic testing for FH, total serum cholesterol level was recorded for 4360 patients. Of these, 956 patients had total serum cholesterol levels above 10.0 mmol/l before lipid-lowering drug therapy was started. They fulfilled the criteria of having FH according to the guidelines of the European Atherosclerosis Society [7].

DNA from 902 of the 956 unrelated patients was available for study. Of the 902 patients a mutation in the LDL receptor gene had been identified in 461 patients (51.1%) by DNA sequencing of the promoter region and the individual exons with flanking intron sequences. A total of 64 different mutations in the LDL receptor gene were identified. In addition, ten patients (1.1%) possessed the mutation R3500Q in the apolipoprotein B-100 gene as determined by the assay of Hansen et al. [26]. The remaining 431 patients participated in this study to compare MLPA and long-range PCR to detect deletions/duplications. Analyses of the five long-range PCRs used in this study, have until now been the standard method in our laboratory to detect large deletions/duplications in the LDL receptor gene [27]. Of the 431 patients, 187 (43.4%) were males and 244 (56.6%) were females. The mean age was 46.7 (±13.1) years and 12.5% had xanthomas. Mean total serum cholesterol level before lipid-lowering drugs were started, was 11.3 (±1.28) mmol/l.

2.2. Long-range PCR

Five amplicons of the LDL receptor gene were analyzed by the Expand Long Template PCR System...
according to the instructions by the manufacturer (Roche Diagnostics, Mannheim, Germany). The amplicons spanned exons 2–6 (7.3 kb), 5–11 (9.0 kb), 8–14 (9.0 kb), 10–16 (14.5 kb) and 12–18 (14.5 kb; Fig. 1). They were generated using primers flanking the indicated exons, except for the 3’ primer used to amplify exons 12–18, which had its 3’ end 1132 bp into exon 18.

2.3. MLPA

The principle for detecting deletions/duplications by MLPA is illustrated in Fig. 1. MLPA kit for the LDL receptor gene (P062 LDLR MLPA kit) was purchased from MRC Holland (Amsterdam, The Netherlands). The probe mix contains probes for 16 of the 18 exons. No probes are present for exons 10 and 13 which are in close proximity to the neighboring exons, with introns 9 and 13 spanning 85 and 136 bp, respectively. Moreover, neither intron 9 nor intron 13 contains any Alu repeats [28]. Thus, the probability of a deletion break point occurring in the two introns, must be low. However, single exon deletions/duplications of exons 10 and 13 cannot be detected by the current version of the kit. As controls, 14 probes for other human genes located on different chromosomes are included in the kit. The assay was conducted according to the instructions by the manufacturer. The fragments were analyzed on a 310 Genetic Analyzer (Applied Biosystems, Foster City, CA) using Genescan-Rox 500 size standard. Data were retrieved by the Genescan software and exported to Microsoft Excel spreadsheet for further analysis of peak heights. Peak heights were normalized by dividing the peak height by the combined peak height of all peaks in that capillary. Deletions were suspected when the peak height was lower than 65% of the controls, and duplications were suspected when the peak height was higher than 135% of the controls. Deletions were confirmed by PCR using exon-specific primers binding to exons flanking the deletion break points. Duplications were confirmed by

Fig. 1. Principle of MLPA to detect deletions and duplication. The principle of how a deletion of an exon can be detected MLPA is shown in the haploid state for a hypothetical gene containing three exons. Two specific probes bind adjacent to each other in each exon. All 5’ probes and all 3’ probes contain primer sequences Y and X, respectively. After the probes have annealed to the target sequences they can be ligated and subsequently amplified using a single primer set with primers binding to sequences Y and X, respectively. To discriminate between PCR fragments amplified from the different exons, a stuffer sequence of variable length is included in the X-probe. If a part of an exon containing one or both probe sequences is deleted, as illustrated by deletion of exon 2 in this figure, the two exon-specific probes for exon 2 are not ligated. Accordingly, they cannot be amplified by PCR. After the exon-specific PCR fragments have been separated by electrophoresis, the amounts of different PCR products are quantitated and normalized by dividing the peak heights of all peaks in the capillary. In the diploid state the wild-type sequence will result in a theoretical value of 2, whereas heterozygosity for a deletion will result in a theoretical value of 1.
PCR using 5′ primers located in the 3′ part of the relevant exon and a 3′ primer located in the 3′ part of the same exon. By this strategy, only duplicated exons will be amplified.

3. Results

Eleven deletions and two duplications in the LDL receptor gene were identified by MLPA in this group of unrelated FH patients with total serum cholesterol levels above 10.0 mmol/l (Table 1). Two representative deletions and one duplication are shown in Fig. 2.

Only six of the eleven deletions detected by MLPA were also detected by one of the five long-range PCRs that spanned the LDL receptor gene. Deletions EX2del, EX2_3del, EX2_4del not detected by long-range PCRs, all had its 5′ deletion break point upstream of primer sequence for the 5′ primer used to amplify exons 2–6. Neither deletions EX7_15del nor EX11_18del could be detected by any of the five long-range PCRs because one or both of the deletion break points were outside the amplified regions.

Deletion EX11_18del could have its 3′ deletion break point within exon 18 downstream of the exon 18 probe binding site, or it could be downstream of the LDL receptor gene. In the latter case even genes downstream of the LDL receptor gene might be deleted. However, exon 9 of the KIAA1518 gene (XM_170889) downstream of the LDL receptor gene that hybridizes to one of the control probes of the MLPA kit, was not deleted as determined by MLPA analysis (Fig. 2b). Thus, the 3′ deletion break point was within the 9.3 kb region between exon 9 of the KIAA1518 gene and the exon 18 probe binding site of the LDL receptor gene.

By using a 5′ primer in intron 9 and a 3′ primer spanning nucleotides 2600–2624 downstream of the LDL receptor gene, a 2.5 kb deletion fragment was observed in patients heterozygous for EX11_18del, but not in normal controls (data not shown). However, no deletion fragment was observed using 3′ primers located between the LDL receptor gene and nucleotide 2305 downstream of the LDL receptor gene. These findings indicate that the 3′ deletion break point is downstream of the LDL receptor gene. Even though the kit does not contain any probe for exon 10, it can be determined that exon 10 is not deleted since several of the patients with EX11_18del were heterozygous for the common polymorphism 1413 G→A [29] in exon 10 (data not shown).

Duplication EX9_10dup was identified both by MLPA (Fig. 2c) and by long-range PCR of exons 8–14 (data not shown). However, the duplication-containing PCR fragment was so faint that it escaped diagnosis in one of the two patients possessing it. Duplication EX7_10dup was not detected by the initial long-range PCR analysis, but could be detected as a very faint band when the gel was re-analyzed after the duplication had first been demonstrated by MLPA. Since only two duplications were identified by MLPA, DNA from additional subjects containing two known duplications within the LDL receptor gene (EX3_6dup, EX3_8dup, kindly provided by Joep C. Defesche, Academic Medical Center, Amsterdam), were run as controls to assess the sensitivity of MLPA to detect duplications. Both duplications were clearly identified (data not shown).

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Deletions and duplications of the LDL receptor gene identified among Norwegian FH patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deletions/ Duplications</td>
<td>Sizea (kb)</td>
</tr>
<tr>
<td>Deletions</td>
<td></td>
</tr>
<tr>
<td>EX2del</td>
<td>3</td>
</tr>
<tr>
<td>EX2_3del</td>
<td>5</td>
</tr>
<tr>
<td>EX2_4del</td>
<td>9</td>
</tr>
<tr>
<td>EX7_8del</td>
<td>3</td>
</tr>
<tr>
<td>EX7_15del</td>
<td>13</td>
</tr>
<tr>
<td>EX9_10del</td>
<td>3</td>
</tr>
<tr>
<td>EX9_12del</td>
<td>6</td>
</tr>
<tr>
<td>EX11_14del</td>
<td>8</td>
</tr>
<tr>
<td>EX11_18del</td>
<td>22</td>
</tr>
<tr>
<td>EX13_15del</td>
<td>6</td>
</tr>
<tr>
<td>EX16_18delc</td>
<td>10</td>
</tr>
<tr>
<td>Duplications</td>
<td></td>
</tr>
<tr>
<td>EX9_10dup</td>
<td>3</td>
</tr>
<tr>
<td>EX7_10dup</td>
<td>4</td>
</tr>
</tbody>
</table>

a As determined by PCR-based methods using primers flanking the deletion break points. The sizes should be regarded as approximations.

b MLPA: multiplex ligation-dependent probe amplification; LR-PCR: long-range PCR.

c Also known as FH-Helsinki.
Fig. 2. Identification of deletions and duplications of the LDL receptor gene by MLPA. Two representative deletions and one duplication in the LDL receptor gene detectable by MLPA are shown after data for peak heights were retrieved, normalized and converted to an Excel spreadsheet. The copy number is shown for 14 control genes and for 16 of the 18 exons of the LDL receptor gene (indicated by Arabic numerals). No probes are available for exons 10 and 13 of the LDL receptor gene. Panel (a) shows a 5 kb deletion of exons 2 and 3. Panel (b) shows a 22 kb deletion of exons 11–18 and panel (c) shows a 3 kb duplication of exons 9 and 10. Since no probe is available for exon 10, duplication of exon 9 only can be identified by MLPA. However, subsequent PCR-based analyses have shown that exon 10 also was duplicated (data not shown). “X” of the control genes represents exon 9 of the KIAA1518 gene downstream of the LDL receptor gene.
Of the 431 index patients screened, deletions or duplications were identified in 44 patients (10.2%) by MLPA (Table 1). If five long-range PCRs had been the only screening method, a deletion would have been missed in 23 of the 44 patients (52.3%).

A total of 127 family members were screened for deletions/duplications identified in the 44 index patients. Of these, 85 family members possessed a deletion and five possessed a duplication. If five long-range PCRs had been the only screening method, a molecular genetic diagnosis would have been missed in 46 of the 90 (51.1%) deletion/duplication positive relatives.

In addition to the 44 index patients possessing deletions or duplications, 471 of the 902 unrelated patients had previously been found to have mutations in the LDL receptor gene or in the apolipoprotein B-100 gene by PCR-based methods. Thus, a total of 515 of the 902 patients (57.1%) with a clinical diagnosis of FH and values for total serum cholesterol above 10.0 mmol/l, have been provided with a molecular genetic diagnosis.

4. Discussion

In this study we have compared MLPA to five long-range PCRs that spanned the LDL receptor gene, to detect large deletions/duplications in the LDL receptor gene. This was studied in a group of 431 unrelated FH patients with total serum cholesterol levels above 10.0 mmol/l. None of the subjects possessed mutations in the promoter region or in the translated parts of the 18 exons with flanking intron sequences detectable by DNA sequencing of the LDL receptor gene. Nor did anyone possess the R3500Q mutation in the apolipoprotein B gene.

Eleven deletions and two duplications were detected by MLPA. Of the deletions, six were also detected by the use of five different long-range PCRs. However, the remaining five deletions could have been detected if additional amplicons had been analyzed. This illustrates that a large number of different amplicons must be studied in order to achieve maximum sensitivity of long-range PCR.

The low sensitivity of long-range PCR to detect duplications in heterozygous patients, is illustrated by the failure of long-range PCR of exons 6–10, to identify EX7_10dup by the initial examination of the gel, and the finding that EX9_10dup was identified in only one of the two patients possessing the duplication.

The failure to detect more than two duplications by MLPA was somewhat surprising since the sensitivity of detecting duplications, should be comparable to the sensitivity of detecting deletions. Moreover, assuming that homologous unequal recombination is the key mechanism for creating large deletions [30], one would expect duplications to be as prevalent as deletions. However, since MLPA analysis also detected the two known duplications, there is no reason to believe that the sensitivity of MLPA of detecting duplications, is lower than the sensitivity of detecting deletions. Apparently, deletions are therefore much more prevalent than duplications in the Norwegian population. This notion is in agreement with data from other populations [31–33].

Whereas false negatives may be a problem with long-range PCR, false positives may be a potential problem with MLPA. As MLPA makes use of two probes that bind adjacent to each other for subsequent ligation, point mutations in the probe-binding sequences may interfere with probe binding or probe ligation. Consequently, the amount of ligated product will be reduced and data analysis may falsely call it a deletion. Thus, all MLPA positive results should be confirmed by an independent method. PCR-based methods are suitable for this purpose, and may also be used to screen at-risk relatives.

When compared to chromosomes 7, 14, 21 and 22, chromosome 19 contains approximately twice the number of Alu repeats [34]. In the LDL receptor gene Alu repeats constitute 65% of the intronic sequences [28]. These Alu repeats are frequently involved in homologous unequal recombination [30]. Of the 45 deletions in the LDL receptor gene that were known as of 1992, the deletion break points have been sequenced in 10. Nine of these had an Alu sequence at one or both break points [35]. Because the major focus for diagnosing FH by molecular genetic methods has been to screen for point mutations and small deletions/duplications by PCR-based methods such as SSCP [12–14], DGGE [17] and DNA sequencing [20], patients with large deletions/duplications may largely have escaped diagnosis. Such mutations may account for 5–15% of the mutations...
causing FH [31–33] which is in agreement with the findings in our study.

The finding that our combined efforts to detect mutations in the LDL receptor gene or in the apoB gene identified a mutation in only 515 of the 902 patients (57.1%), illustrates the low specificity of a clinical diagnosis of FH as well as the existence of other genes than the LDL receptor and apolipoprotein B genes underlying autosomal dominant hypercholesterolemia. Such genes include the PCSK9 gene [36] on chromosome 1p32 that has been found to be a rare cause of autosomal dominant hypercholesterolemia in Norway [37].

One drawback with the current version of the MLPA kit is the lack of probes for exons 10 and 13. Thus, individual deletions/duplications of the two exons cannot be detected. Moreover, PCR-based methods must be used to study whether the two exons are affected by deletions/duplications of neighboring exons. This applies to deletions EX9_10del, EX9_12del, EX11_14del, EX11_18del, and EX13_15del and duplications EX9_10dup and EX7_10dup in our study. Thus, there is a great need for future versions of the MLPA kit to contain probes for exons 10 and 13. The fact that the deletion/duplication break point frequently is located in intron 10, probably reflects the existence of six Alu repeats within this intron [28].

For MLPA to generate high quality data, high molecular weight DNA should be used. Our experience is that MLPA has similar requirements for high quality DNA as long-range PCR. The hands-on time for MLPA is approximately 30–50% lower than for analyzing five amplicons by long-range PCR. In contrast, the direct cost of MLPA is approximately two times higher (12) than the cost of analyzing five amplicons by long-range PCR (6). Moreover, MLPA requires expensive instrumentation for fragment analysis. Even though the direct cost of MLPA is higher, it has replaced long-range PCR for routine diagnostics of FH in our laboratory because of its higher sensitivity and reduced hands-on time.

References

[17] Sheffield VC, Cox DR, Lerman LS, Myers RM. Attachment of a 40-base-pair G+C rich sequence (GC-clamp) to genomic


