methods

# Multiplex ligation-dependent probe amplification of *LDLR* enhances molecular diagnosis of familial hypercholesterolemia

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**Abstract** **Autosomal dominant (AD) familial hypercholesterolemia [FH; Mendelian Inheritance in Man (MIM) 143890] typically results from mutations in the LDL receptor gene (*LDLR*), which are now commonly diagnosed using exon-by-exon screening methods, such as exon-by-exon sequence analysis (EBESA) of genomic DNA (gDNA). However, many patients with FH have no *LDLR* mutation identified by this method. Part of the diagnostic gap is attributable to the genetic heterogeneity of AD FH, but another possible explanation is inadequate sensitivity of EBESA to detect certain mutation types, such as large deletions or insertions in *LDLR*. Multiplex ligation-dependent probe amplification (MLPA) is a new method that detects larger gDNA alterations that are overlooked by EBESA. We hypothesized that some FH patients with no *LDLR* mutation detectable by EBESA would have an abnormal *LDLR* MLPA pattern. In 70 unrelated FH patients, 44 had *LDLR* mutations detected by EBESA, including missense, RNA splicing, nonsense, or small deletion mutations, and 5 had the *APOB* R3500Q mutation. Among the remaining 21 AD FH patients with no apparent *LDLR* mutation, we found abnormal *LDLR* MLPA patterns in 12 and then demonstrated the deleted sequence in 5 of these. These findings indicate that MLPA may be a useful new adjunctive tool for the molecular diagnosis of FH.**—Wang, J., M. R. Ban, and R. A. Hegele. **Multiplex ligation-dependent probe amplification of *LDLR* enhances molecular diagnosis of familial hypercholesterolemia. *J. Lipid Res.* 2005.** 46: **366–372.**

**Supplementary key words** atherosclerosis • genetics • monogenic disorders • DNA analysis • low density lipoprotein receptor gene

Autosomal dominant (AD) familial hypercholesterolemia [FH; Mendelian Inheritance in Man (MIM) 143890] affects ~1 in 500 people and typically results from a mutation in the LDL receptor gene (*LDLR*; MIM 606945) (1). Affected individuals have increased plasma LDL cholesterol, which without adequate diagnosis and intervention can increase the risk of fatal coronary heart disease by up to 100-fold compared with the general population (2). Fortunately, treatment with statin drugs can substantially reduce this risk and improve clinical outcome (2), stressing the importance of early and accurate diagnosis.

At the molecular level, FH is now commonly diagnosed using exon-by-exon screening methods, such as exon-by-exon sequence analysis (EBESA) of *LDLR* from genomic DNA (gDNA) (3, 4). However, this method finds mutations in only ~50% of clinically diagnosed FH patents (3, 4). Part of the diagnostic gap is attributable to the heterogeneity of AD FH (5). For instance, HCHOLAD2 (MIM 144010), which results from a missense mutation in *APOB* affecting the LDL receptor binding domain of apolipoprotein B-100 (apoB-100; MIM 107730) (6, 7), accounts for 5–10% of the AD FH phenotype. A rare FH subtype called HCHOLAD3 (MIM 603776) results from mutations in *PCSK9* (MIM 607786), encoding neural apoptosis-regulated convertase-1 (5, 8, 9). A similarly rare autosomal recessive FH subtype called HCHOLAR1 (MIM 603813) results from mutations in *ARH* (MIM 605747), encoding a putative adaptor for the LDL receptor (10). However, even after accounting for genetic heterogeneity, many clinically diagnosed FH patients have no *LDLR* mutation with EBESA.

Another possible explanation for the gap in FH molecular diagnosis is inadequate sensitivity of EBESA to detect certain mutation types, such as large deletions or insertions. The larger gDNA alterations create effective hemizygosity for single exons and result in an EBESA profile that is indistinguishable from homozygosity for two normal *LDLR* alleles. Multiplex ligation-dependent probe amplification (MLPA) is a new analytical method (11) that detects larger gDNA deletions or insertions that would otherwise be overlooked by EBESA (12). We hypothesized that some FH patients with no *LDLR* mutation detectable by EBESA would have an abnormality detectable using MLPA. Of 70 unrelated FH patients, 44 had *LDLR* mutations detected by EBESA, including missense, RNA splic-

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ing, nonsense, or small deletion mutations. Five had the *APOB* R3500Q mutation. Among the remaining 21 AD FH patients with no apparent *LDLR* mutation, we found abnormal *LDLR* MLPA patterns in 12 and confirmed the deleted sequences in 5. MLPA may be a useful new adjunctive tool for the molecular diagnosis of FH.

## METHODS

### Study subjects

We studied 70 unrelated Caucasian patients from southwestern Ontario who had each been referred to a tertiary lipid clinic for diagnosis and treatment of hypercholesterolemia. Each subject had a clinical diagnosis (made by one clinician) of FH according to validated clinical and biochemical criteria (7). This study had the approval of our ethics review panel; all subjects gave informed consent.

### DNA sequence analysis

From gDNA extracted with the Puregene kit (Gentra Systems, Minneapolis, MN), EBESA of the *LDLR* was performed as described (4) by sequencing both strands of the promoter region and of the translated parts of all 18 exons (primer information and sequences available upon request). In addition, *APOB* exon 26, which harbored the receptor binding domain of apo B-100, including the recurrent R3500Q mutation in HCHOLAD2, was examined in all patients using direct sequence analysis. Standard DNA sequencing reactions using the Big Dye Terminator cycle sequencing kit version 3.1 (PE-Applied Biosystems, Mississauga, Ontario, Canada) were analyzed using a 3730 Automated DNA Sequencer (PE-Applied Biosystems) according to the protocols of the London Regional Genomics Centre (www.lrgc.ca).

### MLPA reaction

The SALSA P062 *LDLR* MLPA kit was obtained from MRC-Holland (Amsterdam, the Netherlands). The principles and stages of MLPA have been previously described (11). Briefly, each pair of diagnostic probes is designed such that the probes hybridize immediately adjacent to each other on the same gDNA target in preparation for a subsequent ligation step. Each probe consisted of a target-specific sequence and either a common forward or reverse primer-specific sequence. One probe from each pair contained a "stuffer" sequence of 19–364 nucleotides (nt), which was unique and thus diagnostic for the particular probe pair. The *LDLR* MLPA kit contained 31 sets of probes, 16 of which were *LDLR* specific and the others were control standards. Reactions were carried out in 200 μl tubes in a model 9700 thermocycler (PE-Applied Biosystems). One hundred to 300 ng of gDNA from each subject was diluted in 5 μl of 1× Tris-EDTA and denatured at 98°C for 5 min. MLPA buffer and probe mix (1.5 μl of each) were then added to allow probes to anneal to target gDNA by heating at 95°C for 1 min and incubating at 60°C for 16 h. Annealed probes were ligated at 54°C for 15 min followed by inactivation at 98°C for 5 min. Ten microliters of ligation reaction was removed for multiplex amplification using a pair of common primers, of which one was labeled with the fluorescent dye FAM. *Taq* polymerase was added to the reaction at 60°C, followed by 33 cycles of 95°C for 30 s, 60°C for 30 s, 72°C for 1 min, and a final extension step of 72°C for 20 min. Two microliters of reaction solution was used for fragment analysis on the 3730 capillary sequencer, with LIZ-500 size standards (PE-Applied Biosystems). The procedure was performed according to the manufacturer's instructions (www.mrc-holland.com).

### MLPA fragment analyses

Data analysis was performed using GeneMapper version 3.5 (PE-Applied Biosystems). Electropherograms of fragments from MLPA analysis of *LDLR* from normal subjects show a profile composed of 31 peaks (range, 130–382 nt). Exons 10 and 13 are not interrogated with the current version of the probe set. The multiplex contained five internal DNA quantity (DQ) control fragments. Four of these, of 64, 70, 76, and 82 nt, were ligation independent and were included to demonstrate that sufficient template DNA was present for the entire multiplex amplification reaction. The fifth DQ fragment, of 94 nt, indicated successful ligation by producing a peak of comparable size to that of the other chromosome-specific probes in the multiplex. The relative areas under the curve (AUCs) for peaks in each sample were determined. The relative peak AUC for each probe was calculated using four adjacent peaks, two on either side, as internal controls. The fraction of each peak was then divided by the median peak fraction of the corresponding fragment from five normal control samples. In 18 normal individuals, these calculations gave values close to 1.0, which corresponded to the normalized mean peak area and standard deviations for an individual with two copies of the target sequence. Copy number results $>1.33$ or $<0.75$ were flagged. Calculations were performed on samples processed within an assay run. Two-sample *t*-tests were used for all statistical comparisons against the profiles of normal samples, with nominal $P < 0.05$.

### DNA sequence confirmation of deletion break point

As proof of the principle that a reduced MLPA peak in this study sample corresponded to a deleted region of gDNA, we identified five patients with a similar MLPA pattern, each showing ~50% loss of height of exon 1. All of these patients had French surnames, leading us to consider that this particular MLPA pattern might have resulted from the >15 kb deletion (Δ>15 kb) at the 5′ end of the *LDLR*. This deletion is the most common cause of FH in French Canadians (1, 13). We used the reported method (13) to sequence the deletion break points in these five patients. Briefly, primer pairs 23F (5′-CAC ATG CTC GAG TGT AAG AAC-3′) and 17R (5′-GGT TGG CGT TTT TCA TAT TAG ACC-3′) were used to amplify a 435 bp fragment spanning the deletion break point. When present, this fragment was amplified and sequenced. Primers 20F (5′-AGA CCC AGA AAG AAA TGA TTC G-3′) and 20R (5′-GAA GTG TCC TTG TCT TCT CAT CG-3′) produced a 236 bp fragment, which indicated the presence of the wild-type allele (13), confirming heterozygosity.

## RESULTS

### Baseline attributes of the study sample

Among the sample of 70 FH patients, 35 (50%) were women and the age (mean ± SD) was 41.2 ± 16.5 years. The mean ± SD ages for men and women were 43.4 ± 17.4 years and 38.5 ± 15.5 years, respectively. Mean ± SD values for plasma LDL cholesterol were 7.15 ± 1.68 mmol/l, 7.34 ± 1.79 mmol/l, and 6.97 ± 1.56 mmol/l for the overall sample, men, and women, respectively. Thirty-four percent of patients had xanthomata and 22% had xanthelasmata.

### Mutations in FH patients found by EBESA

Twenty-one FH patients (30% of the sample) were heterozygous for a total of 18 different *LDLR* missense muta-

tions, namely A29S, C42R, E80K, E119K, C163Y, D203N, E207K, G314S, Q363P, A370T (three patients), R395W, V408M, T413M, Y421C*, L458P, L561P (two patients), E760D, and N804K. Twelve FH patients (17.1% of the sample) were heterozygous for a total of seven different RNA splicing mutations, namely IVS2 donor +2T>G*; IVS3 donor +1G>A (two patients), IVS3 acceptor −2A>C, IVS7 acceptor −8T>C (three patients), IVS8 acceptor −10G>A, IVS12 donor +15C>A*, and IVS14 donor +5G>A (three patients). Two FH patients (3% of the sample) were each heterozygous for an in-frame deletion, namely ΔAsp36 and ΔGly198*. Nine FH patients (12.9% of the sample) were heterozygous for a total of seven different nonsense mutations, namely W23X, fs R103P-X108, C152X, fs G197V-X243, fs F381-X418, fs K497L-X528*, and C660X (three patients). Among the mutations listed above are five newly discovered *LDLR* mutations, which are indicated by asterisks. All *LDLR* mutations were absent from the genomes of 100 healthy control subjects. Five FH patients (7.1% of the sample) had the *APOB* R3500Q mutation. Twenty-one FH patients (30% of the sample) had no *LDLR* mutation by EBESA and did not have the *APOB* R3500Q mutation.

### MLPA analysis

The 21 FH patients who had neither a mutation in *LDLR* by EBESA nor the *APOB* R3500Q mutation were studied using *LDLR* MLPA. In addition, *LDLR* MLPA was performed on gDNA from 23 healthy normal subjects. *LDLR* deletion mutations were apparent on qualitative inspection of MLPA peak profiles. This is illustrated in **Figs. 1**, **2**, in which comparison of peak heights of a probe located within *LDLR* and neighboring control peaks indicated a gene dosage mutation. Normalized peak areas for exons were determined using the normal samples as described in Methods. Standard deviations of <7% of the normalized mean were recorded for all samples using the means of the relative copy numbers of deletion/duplication region probes. We set threshold values for the identification of abnormal results at 0.75 and 1.33 (corresponding to >4 standard deviations from the mean) (**Table 1**). Among the 21 previously mutation-free FH patients, there were seven abnormal MLPA patterns seen in 12 patients. Four involved reduced peaks for one exon only: Δexon 1 (five patients), Δexon 2, Δexon 3, and Δexon 6. Three involved reduced peaks for consecutive exons: Δexon 1–6 (two patients), Δexon 2–6, and Δexon 4–14. Control MLPA profiles of three FH patients who had missense mutations in *LDLR* were all normal (data not shown).

### DNA sequence break point confirmation in FH patients with abnormal MLPA

To confirm that an abnormal MLPA pattern corresponded to a specific gDNA sequence change, we amplified a gDNA fragment that harbored the deletion break point. There is no procedure at present to select primers to amplify deleted gDNA regions, especially when the deleted region was just approximately characterized with MLPA, itself a relatively novel method. We noted that the five subjects with the MLPA pattern corresponding to Δexon 1 each had French surnames. This led us to consider that this abnormal MLPA pattern might have been the result of the Δ>15 kb deletion at the 5′ end of the *LDLR* that is the most prevalent cause of FH in French-Canadian patients (13). We used the reported method to detect Δ>15 kb in these five patients (13). We first found that each had the diagnostic 435 bp band that could only be amplified if the deletion was present (13), that each was heterozygous for this mutant fragment, and that the sequence of this fragment corresponded to Δ>15 kb (13) in all five subjects (**Fig. 3**).

### Biochemical attributes according to molecular diagnosis

We stratified patients into the following molecular subgroups: *1*) heterozygotes for missense mutations; *2*) heterozygotes for splicing mutations; *3*) heterozygotes for either nonsense mutations or in-frame deletions; *4*) abnormal MLPA pattern; *5*) heterozygotes for *APOB* R3500Q; and *6*) no *LDLR* mutation and normal *APOB* receptor binding domain sequence (Table 1). The mean LDL cholesterol among the small group of 12 FH patients with abnormal *LDLR* MLPA patterns was not different from that of the other 58 patients (7.98 ± 2.36 mmol/l versus 6.98 ± 1.47 mmol/l; $P = 0.19$ by Student's *t*-test using unequal variances).

## DISCUSSION

The principal novel findings of this report are as follows: *1*) approximately one-third (21 of 65) of FH patients without the *APOB* R3500Q mutation had no *LDLR* mutation detected by EBESA of gDNA; and *2*) approximately one-half (12 of 21) of FH patients with no *LDLR* mutation detected by EBESA had an abnormal *LDLR* MLPA pattern. Five of 70 (7.1%) FH subjects had the *APOB* R3500Q mutation, which is consistent with results from other study samples (6). The abnormal MLPA pattern in five patients was confirmed by sequencing to correspond to a specific deletion, namely Δ>15 kb, at the 5′ end of *LDLR*. Although *LDLR* missense mutations are most common in FH, MLPA abnormalities, mainly deletions, also appear to be prevalent. These results indicate that MLPA might be a promising method to detect additional gDNA abnormalities of the *LDLR* gene.

Technology is an important determinant of the mutation type detected. For instance, in the pre-PCR era, the most prevalent *LDLR* mutation type in FH was large-scale gDNA rearrangement, such as large insertions and deletions (14). One reason for this was the relative enrichment within *LDLR* of sequences that were predisposed to imperfect recombination events or exon shuffling (14). But another reason was the fact that most *LDLR* mutations in the late 1980s were detected by Southern blotting. Southern blots were ideal for detecting large-scale gDNA rearrangements involving >200 nt of target gDNA, which was the resolution limit for DNA fragments. However, detection of small mutations was beyond the Southern blot's

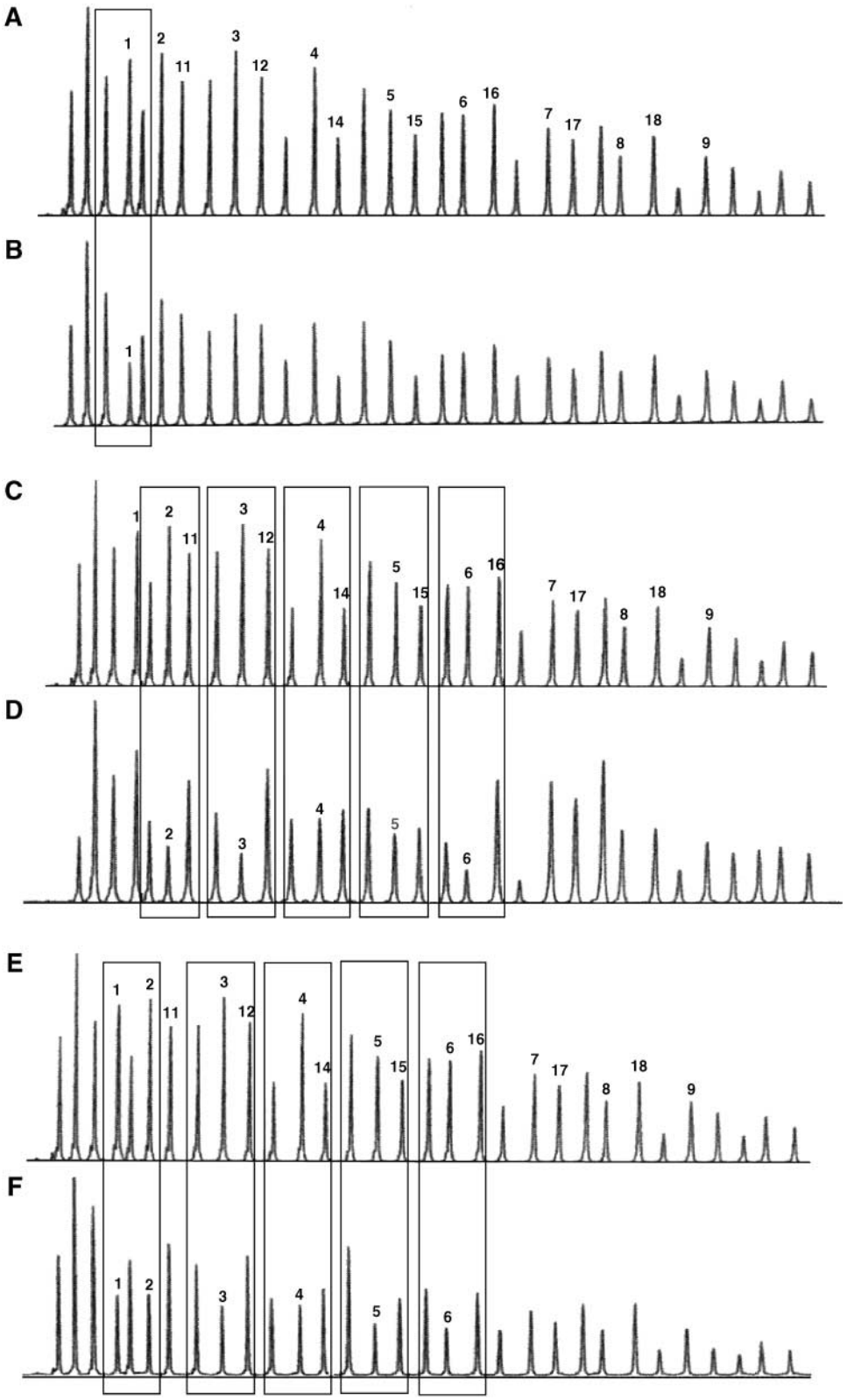


**Fig. 1.** Multiplex ligation-dependent probe amplification (MLPA) electropherogram tracings from normal and familial hypercholesterolemia (FH) subjects. A, C, E: Tracings of LDL receptor gene (*LDLR*) peaks from a healthy subject. The peaks corresponding to the *LDLR* exons are indicated with numerals. Exons 10 and 13 are not interrogated by the current set of probes. B: Abnormal MLPA electropherogram tracing from a subject with FH. The box highlights the loss of area under the curve (AUC) for the exon 1 peak, which was quantified at 0.48 of the mean AUC for exon 1 from normal subjects (see Fig. 2), indicating a loss of gene dosage that followed Mendelian stoichiometry. DNA sequence analysis showed that this subject was heterozygous for the >15 kb deletion (Δ>15 kb; see Fig. 3). D, F: Reductions of peaks for exons 2–6 and exons 1–6, respectively, for the exon peaks from normal subjects (aligned by boxes). Plots of the normalized peak areas for the FH patients in B, D, and F are shown in Fig. 2, A, G, and F, respectively.

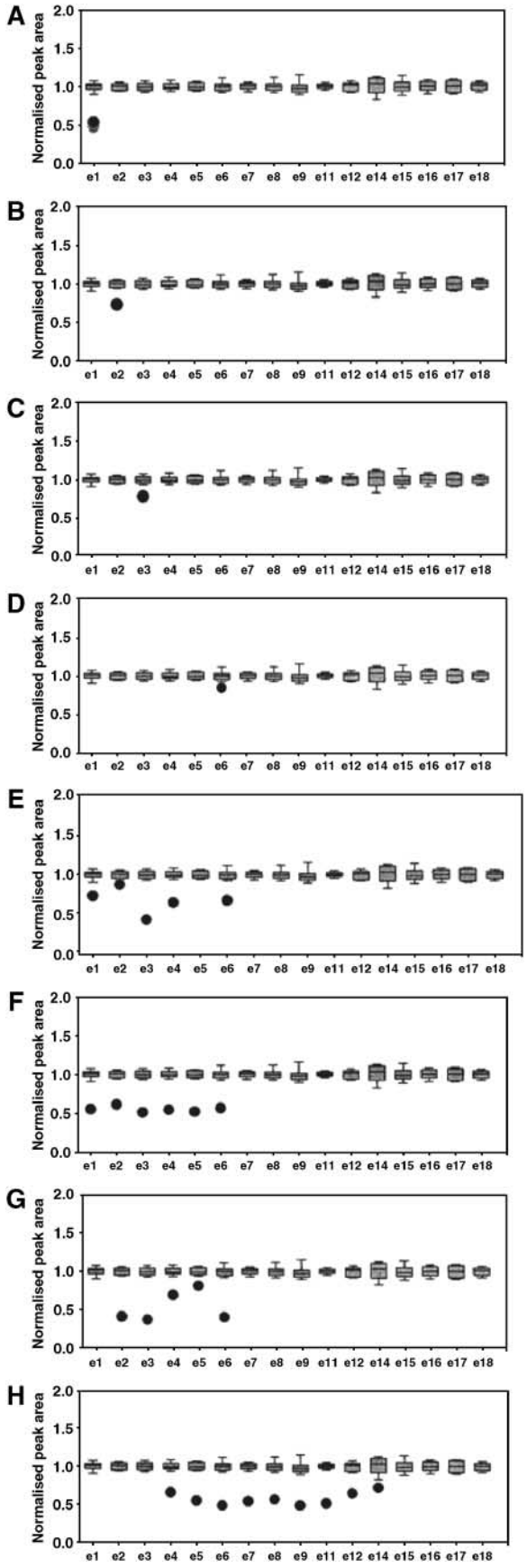


**Fig. 2.** Normalized peak areas in normal subjects and results from FH subjects. Exon numbers are shown by "eN" (where N is the number of the exon) along the abscissa. For box plots of normalized peak AUC for *LDLR* exons from MLPA from 18 normal controls, means (vertical lines) and standard deviations (error bars) are shown. The dots represent AUCs for individual FH subjects by MLPA according to the exon. An individual AUC value was flagged if it was $<0.75$ or $>1.33$ compared with the normalized mean, and only areas that fell outside this range are shown. A: Combined data from five FH subjects whose MLPA tracings had markedly low peak areas for exon 1, a pattern that we designated as Δexon 1. The genomic DNA (gDNA) from these five subjects was sequenced (see Fig. 3). B: MLPA tracing from a FH subject with a markedly low peak area for exon 2, a pattern that we designated Δexon 2. C: MLPA tracing from a FH subject with a low peak area for exon 3, a pattern that we designated Δexon 3. D: MLPA tracing from a FH subject with a markedly low peak area for exon 6, a pattern that we designated Δexon 6. E, F: MLPA tracings from FH subjects with markedly low peak areas for exons 1 to 6, designated Δexon 1–6. G: MLPA tracing from a FH subject with markedly low peak areas for exons 2 to 6, designated Δexon 2–6. H: MLPA tracing from a FH subject with markedly low peak areas for exons 4 to 14, designated Δexon 4–14. Patient normalized peak plots shown in A, G, and F correspond to patient MLPA tracings in Fig. 1, B, D, and F.

TABLE 1. Attributes of the study sample according to molecular diagnosis

| Mutation Type | Number | Age | Percent Female | Untreated LDL-C |
|---|---|---|---|---|
| | | *years* | | *mmol/l* |
| Missense | 21 | 42.1 ± 16.8 | 47.6 | 6.74 ± 1.30 |
| Splicing | 12 | 53.9 ± 13.3 | 75.0 | 7.33 ± 0.86 |
| Nonsense plus in-frame deletions | 11 | 30.6 ± 13.4 | 45.5 | 7.54 ± 1.83 |
| Abnormal MLPA pattern | 12 | 34.7 ± 18.7 | 50.0 | 7.98 ± 2.34 |
| *APOB* R3500Q | 5 | 39.6 ± 14.3 | 20.0 | 6.46 ± 1.23 |
| No abnormality | 9 | 44.4 ± 10.3 | 44.4 | 6.70 ± 2.04 |

*APOB*, gene encoding apolipoprotein B; LDL-C, plasma concentration of low density lipoprotein cholesterol; MLPA, multiplex ligation-dependent probe amplification.

resolving capacity, unless a mutation altered a recognition site for a restriction endonuclease. Because Southern blotting was optimal for detecting large-scale *LDLR* rearrangements, by the early 1990s these represented a large proportion of FH mutations (1, 14).

PCR made it easier to quickly detect small mutations from gDNA using relatively few resources and with less expense, effort, and radiation requirements than Southern blotting. Thus, most of the ~1,000 *LDLR* mutations in FH that are in current databases are of the single-nucleotide variety discovered by exon-by-exon PCR-based screening methods such as EBESA (http://www.ucl.ac.uk/fh/). However, PCR-based methods of interrogating gDNA are practically limited to regions of 200–700 bp. A large deletion affecting one *LDLR* allele may never be detected by PCR, because there is no target sequence upon which amplification primers can anneal. Only the normal allele is amplified, creating effective hemizygosity for that region of gDNA. Analysis of the products of such a reaction in the search for a DNA change, whether indirectly (15) or by EBESA, will yield a false-negative result.

MLPA optimally detects alterations over a relatively large gDNA region but uses no radioactivity and is PCR based. As a semiquantitative method, internal standards and controls are required for each analysis, but this need is met by the high throughput afforded by rapid, automated fragment detection. Our cost for *LDLR* MLPA analysis per patient sample, including reagents, duplicate analyses, controls, and labor, was ~$120 US, which seems cost-effective considering the increment in molecular diagnosis. To date there is no optimal procedure for data normalization and for detection of abnormal data points for MLPA (16). For future clinical applications, performance of *LDLR* MLPA will need to be assessed by blinded analysis of FH samples that have well-defined gDNA rearrangements. Various reference samples, including healthy controls and subjects with other mutation types, will need to be assessed, as will intraobserver and interobserver variability. It was promising that we sequenced the deletion break points at the 5′ end of *LDLR* in five subjects whose MLPA analysis showed the Δexon 1 pattern.

MLPA reduced the percentage of FH patients who had no apparent abnormality in gDNA to 12.9% (9 of 70). What is the basis of the FH phenotype in the remaining subjects? Some of these patients might have had other *LDLR* mutation types, such as mutations deep within introns or beyond the region of the promoter and 3′-untranslated regions that could be accessed by our EBESA and MLPA primers. Alternatively, some patients may have had another genetic cause for their FH phenotype, as exemplified by the five patients with *APOB* R3500Q. We used EBESA to examine *PCSK9* (MIM 607786) and *ARH* (MIM

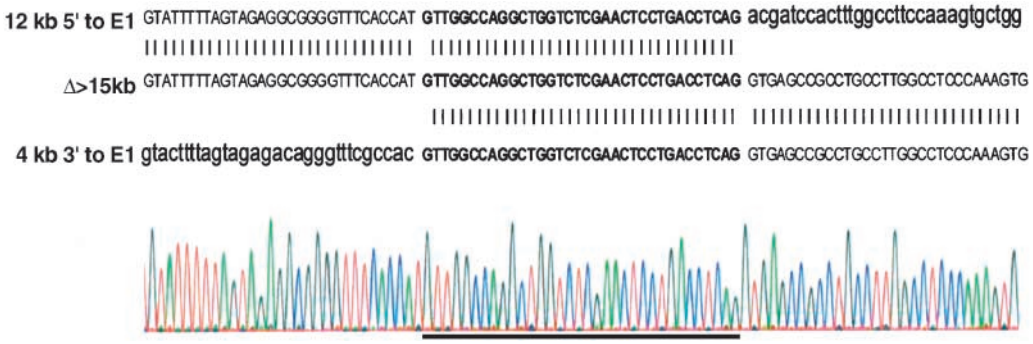


**Fig. 3.** DNA sequence analysis from a subject with abnormal MLPA from Figs. 1B and 2A. Sequence electropherogram tracing of the fragment spanning the deletion break point in the FH subject is shown at bottom. Normal gDNA sequences from 12 kb upstream of exon 1 and from 4 kb downstream of exon 1 are shown in the first and third lines of alphabetic text, respectively. The sequence corresponding to the electropherogram tracing of the patient's DNA is the middle line of alphabetic text. The mutant sequence is identical to the normal region 12 kb upstream of exon 1, followed by a 34 nucleotide common sequence found both 12 kb upstream and 4 kb downstream of exon 1, followed by normal sequence found 4 kb downstream of exon 1. The common sequence is highlighted by a line underneath the electropherogram tracing and is shown in upper case characters in each alphabetic line of sequence. The normal sequence that has been lost from the mutant allele is shown in lower case characters in the alphabetic sequences 12 kb upstream and 4 kb downstream of exon 1. The mutant allele sequence is identical to the Δ>15 kb allele that is the most common *LDLR* in French-Canadian subjects with FH. This sequence was found in all five subjects who showed the MLPA pattern in Figs. 1B and 2A.

605747) in the nine FH patients with no *LDLR* mutation and normal *APOB* exon 26 sequence and found no mutations in these genes (data not shown). Mutations in *PCSK9* and *ARH* might still be found in some of these FH patients through MLPA. Alternatively, causative mutations in some of these FH patients may be found in other genes.

In summary, we report abnormal MLPA patterns in a substantial proportion of FH patients who had neither a *LDLR* mutation using EBESA nor the *APOB* R3500Q mutation. MLPA increased the *LDLR* mutation detection rate in FH subjects with no *APOB* R3500Q mutation from 67.6% to 86.2% (from 44 of 65 to 56 of 65). If this increment in mutation detection can be replicated, MLPA might become a useful adjunct to existing screening methods for the molecular diagnosis of FH. Before MLPA can be applied clinically, it requires formal evaluation of such performance criteria as sensitivity, specificity, and reproducibility. However, the clinical importance of a proper FH diagnosis (2) supports the need to develop sensitive molecular diagnostic algorithms to optimally diagnose FH. The data reported here also suggest the possibility of additional molecular heterogeneity of FH.

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