Abstract We used the single-strand conformational polymorphism method to screen 311 patients with familial hypercholesterolemia from London lipid clinics and Southampton and South West Hampshire health district for mutations in the 3' part of exon 4 of the low-density lipoprotein (LDL) receptor gene. This part of the gene codes for repeat 5 of the binding domain of the LDL receptor, which is known to be critical for the receptor-mediated removal of both triglyceride-rich lipoprotein remnants and LDL. Six previously described mutations were identified in 29 apparently unrelated individuals (9.3%), with the mutations all lying within a 50-bp fragment of the gene. Three of the mutations are null alleles producing no protein, and the other three lead to production of a defective protein. The effect of the different gene mutations on lipid levels was examined, after the data were combined with information on previously reported mutations in this patient group. Mean LDL cholesterol levels were highest in those individuals with a mutation creating a null allele (9.54 mmol/L) and were similar to levels in those individuals with a mutation affecting repeat 5 that resulted in the production of a defective protein (9.37 mmol/L). In this sample, previously identified patients with a defective protein mutation outside repeat 5 had lower mean levels of LDL cholesterol (7.78 mmol/L), which were similar to levels seen in patients in whom the specific mutation had not been identified (7.31 mmol/L). Overall, these differences were highly statistically significant (P<.001). These data reinforce the observations of other researchers that specific mutations in the LDL receptor gene are associated with different effects on plasma lipids and indicate that the phenotype is influenced by the genotype.

Key Words • single-strand conformational polymorphism • LDL receptor mutations • familial hypercholesterolemia • genotype-phenotype

Familial hypercholesterolemia (FH) is caused by mutations in the low-density lipoprotein (LDL) receptor gene. To date, it has been difficult to examine whether specific LDL receptor gene mutations show a different genotype-phenotype relation except in founder populations in which large numbers of carriers for a particular mutation can be found. In such populations, recent studies have suggested that different mutations have different phenotypes such as lipid levels, expectation of clinical sequelae, and drug responsiveness. However, because of their common origin, the patients may also share other genetic factors, and these comparisons may thus be confounded. Here, we report the first attempt to undertake such a study in a group of FH patients from a population with a complex spectrum of LDL receptor gene mutations by combining the characterized LDL receptor gene mutations into functional groups and examining the differences on baseline lipid levels among these groups.

Five classes of mutations at the LDL receptor locus have been identified on the basis of the phenotypic behavior of the mutant protein. Class 1 mutations fail to produce any immunoprecipitable protein (null alleles). The other four classes all produce defective proteins and are affected at different levels of the receptor pathway because of the precise location of the missense mutation. The first 292 residues of the receptor contain 7 imperfect repeats of 40 amino acids that make up the binding domain. Mutational analysis of the binding domain has shown that repeat 1 is not required for binding of either apolipoprotein B (apoB) or apoE, but repeat 5 is required for both ligands. Mutations affecting this part of the protein are thus likely to have a severe effect on the binding function of the receptor. Repeats 2 and 3 as well as 6 and 7 are required for maximal binding of LDL via apoB but not for very-low-density lipoprotein (VLDL) via apoE, and analysis of a naturally occurring mutation in repeat 4 has shown that the substitution of Leu for Ser at position 156 (S156L), which abolishes the binding of LDL but not that of β-VLDL, causes only a mild reduction of receptor function in vitro. The binding domain of the LDL receptor is coded by exons 2 through 6 of the gene, with the 3' end of exon 4 coding for the apoE/apoB binding repeat 5 (amino acids 172 to 210), and both we and others have observed that mutations occur frequently in this part of the gene. It has been suggested that the high mutation rate observed in this part of the gene is caused by a selection bias, because individuals with such mutations are more severely affected than individuals with mutations in...
other parts of the gene. To examine for possible differences of effect of mutations on lipid phenotype, we have compared patients with any mutation causing a null allele with those in whom a missense mutation occurs in repeat 5 and those with a missense mutation elsewhere in the gene. Comparisons were made by combining data from the current screening of the 3' part of exon 4 of the gene with results from previous studies on this group of patients in whom gross deletions or missense mutations in exon 3·5 or exon 14·4 had been detected.

Methods

Selection of Patients

A total of 311 apparently unrelated patients with heterozygous FH were studied. They were from four Lipid Clinics in London (Hammersmith Hospital, Charing Cross Hospital, St. Mary's Hospital [n=189] and the Department of Medicine, University College of London Medical School [n=72]). The patients from the first three clinics have been described in earlier studies.9·10·11 In addition, 50 patients were recruited from Southampton and South West Hampshire health district (I.N.M.D., unpublished data). Standard diagnostic criteria for FH were used,1·2·12 including a serum total cholesterol of more than 7.5 mmol/L and plasma level of LDL cholesterol higher than 4.9 mmol/L with tendon xanthomas in the patient, and a first- or second-degree relative less than 55 years of age with tendon xanthomas and/or definite myocardial infarction. Cholesterol and triglyceride analyses were by laboratories participating in national quality-control schemes. LDL cholesterol was calculated according to Friedewald et al.15 Patients who were found to carry the mutation for familial defective apoB were excluded.16 The 30 patients showing no single-strand conformational polymorphism (SSCP) in the 3' part of exon 4, who were selected for sequencing, all had tendon xanthoma as well as the full standard diagnostic criteria.

Amplification of Genomic DNA

Parts of exon 4 were amplified by polymerase chain reaction (PCR) using two sets of oligonucleotide primer pairs. For the 3' end, primer 1 from bp 528 to bp 547 in exon 4 and primer 2 in intron 4 (5'-GGGACGACGGAGGTAGTATAGGAC-3') were used giving a 236-bp fragment (designated fragment 1). The whole of exon 4 (designated fragment 2) was amplified using primer 3 (5'-AA GTG CAC GGT CTC GGG CAT C AT C CCT G-3') from intron 3 (including Sal I cutting site, as that primer was found to give the most consistent results) and 5' biotinylated primer 2. Oligonucleotides were obtained from Severn Biotech Ltd. The amplifications were performed in an automated thermal cycler (Cambo) with Taq DNA polymerase (GIBCO BRL) in the buffer recommended by the manufacturer and a total volume of 25 μL for fragment 1 and 50 μL for fragment 2. The conditions for both fragments were 95°C for 1 minute and 68°C for 6 minutes for 30 cycles, with 15 second intervals between cycles for fragment 1 and 1 minute intervals for fragment 2.

Direct Sequencing

For sequencing, biotinylated PCR fragment 2 was captured onto Dynal-beads (Dynal UK Ltd) for purification. The single strands were separated by denaturing in NaOH according to the manufacturer's protocol, and the DNA was then sequenced using the Sequenase kit (version 2.0, United States Biochemicals) following the manufacturer's protocol. The oligonucleotides used for sequencing were primer 4 from bp 606 to 588, primer 5 from nucleotides 534 to 552, primer 6 from the intron 4-exon 4 boundary (antisense), and primer 7 from nucleotide 685 to 704.

ApoE Genotyping

ApoE genotype was determined by PCR and an Hha I enzyme digest as previously described.18

Statistical Analysis

Analysis of the pretreated plasma lipid levels and age at the time of diagnosis was carried out using the SPSS/PC+ computer programs (Northwestern University) and STATGRAPHICS II (Statistical Graphics Corp). Plasma levels of triglycerides and lipoprotein(a) were log10 transformed before statistical analysis. Means of plasma lipid levels among groups were compared by ANOVA or Student's t test, both with and without adjustment for age and gender by multiple linear regression. Statistical significance was taken at a value of P<.05. For many patients, data on weight at the time of measurement of pretreatment lipid levels were unavailable, so adjustment for body mass index was not possible. In general, FH patients are not obese, and in samples from the general population adjustment for body mass index, with the normal range, has only moderate effects on cholesterol levels. In addition, no adjustment was made for possible differences in lipid measurements from the different participating clinics. Such differences are likely to be small (<5%), and because each clinic contributed roughly equally to the patients identified with each class of mutations, this is unlikely to have introduced a significant bias.

Results

Identification of Mutations by SSCP

Samples from all 311 FH patients in this study were analyzed by SSCP at the 3' region of exon 4 of the LDL receptor gene. Several combinations of conditions were used, including either 4°C or room temperature with a 4.5% polyacrylamide gel (with and without glycerol). The clearest results were obtained by changes in the dodecyl sulfate and 10 mmol/L EDTA. Five microliters of this dilution was mixed with 5 μL formamide dye (95% formamide, 20 mmol/L EDTA, 0.05% bromphenol blue, 0.05% xylene cyanol FF). The PCR DNA was denatured by boiling for 3 minutes and then put immediately on ice. Samples (4 μL) were loaded onto a 4.5% polyacrylamide nondenaturing gel (ratio of acrylamide to bisacrylamide, 49:1) in 0.089 mol/L Tris-borate, 0.002 mol/L EDTA buffer, with or without 10% glycerol. Gels were 40 cmx30 cmx0.4 mm. Electrophoresis was at 20 mA for 16 hours at room temperature in the case of the 4.5% polyacrylamide gels with 10% glycerol and at 45 mA for 3 hours at +4°C in the case of the 4.5% gel without glycerol. The gels were then transferred onto Whatman 3MM chromatographic paper and dried and exposed to hyperfilm β max (Amersham) for 12 to 24 hours at −70°C before developing.

Identification of Mutations by SSCP

Samples from all 311 FH patients in this study were analyzed by SSCP at the 3' region of exon 4 of the LDL receptor gene. Several combinations of conditions were used, including either 4°C or room temperature with a 4.5% polyacrylamide gel (with and without glycerol). The clearest results were obtained when samples were run in a 4.5% polyacrylamide gel and 10% glycerol at room temperature for 18 hours; Fig 1 shows the SSCP pattern obtained for the six mutations detected. Complete sequencing (not shown) demonstrated that all of these mutations had previously been identified in this region of the gene, either in a subset of the patients analyzed here,10 or in the Dallas collection (E207X).11 The mutations are easily detected by changes in the upper single-strand migration pattern. The smallest mobility shift (0.5% or 1 mm on a 220-mm-long run) was seen for the Cys to stop at codon 210 (designated
patients with any detected mutation had unadjusted total cholesterol and LDL cholesterol levels that were 15% and 22% higher, respectively (P<.0001), and triglyceride levels 18% lower (P<.05) compared with the rest of the FH patients with no detected mutation. This difference was also highly significant (P<.0001 and P<.05, respectively) when the analysis was performed on sex- and age-adjusted values (data not shown).

Among the patients, the group with a mutation causing a null allele had the highest mean total and LDL cholesterol levels, with very similar levels being seen in patients with a defective protein caused by a mutation affecting repeat 5. By contrast, patients with a defective protein mutation outside repeat 5 had mean total and LDL cholesterol levels that were significantly lower and were similar to levels seen in the group of patients with an unknown mutation.

To examine the possibility that the common apoE variation might explain the differences in plasma lipids, we determined apoE genotype. The frequency of the apoE alleles did not differ between those patients with a mutation in repeat 5 compared to the rest of the FH patients (E2, 0.054 versus 0.047; E3, 0.786 versus 0.774; and E4, 0.160 versus 0.179, respectively) nor between those patients in the subgroups of null alleles and defective proteins (data not shown).

Fig 3 shows the distribution of serum cholesterol with age (untreated lipid levels at age of diagnosis) in all heterozygous FH patients in whom the mutation had been identified, including those previously described from this sample, with patients grouped according to the predicted class of mutation of either any defective protein or any null allele. It can be seen that patients with a defective protein (Fig 3a) display a statistically significant correlation of plasma cholesterol with age, whereas there is no statistically detectable correlation in the group with null alleles (Fig 3b). The slope of the regression line for the defective allele group is 0.8 mmol/L per decade.

Discussion

In our hands, SSCP is an effective and rapid method for detecting sequence changes in PCR-amplified DNA.
Comparison of Unadjusted Lipid Levels Between Various Groups of Probands With Familial Hypercholesterolemia

<table>
<thead>
<tr>
<th></th>
<th>Whole Group</th>
<th>Any Mutation</th>
<th>Null Mutations</th>
<th>Defective Protein</th>
<th>Defective Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n</td>
<td>n</td>
<td></td>
<td>Repeat 5</td>
<td>Excluding Repeat 5</td>
</tr>
<tr>
<td><strong>Male/female</strong></td>
<td>75/43</td>
<td>25/19</td>
<td>5/7</td>
<td>11/6</td>
<td>7/4</td>
</tr>
<tr>
<td><strong>Age, y</strong></td>
<td>44.9 (14.5)</td>
<td>41.1 (12.8)</td>
<td>40.1 (11.2)</td>
<td>41.4 (11.1)</td>
<td>42.0 (16.7)</td>
</tr>
<tr>
<td><strong>TC, mmol/L</strong></td>
<td>9.37 (1.77)</td>
<td>10.74 (1.90)§</td>
<td>11.31 (2.11)§</td>
<td>11.23 (1.65)§</td>
<td>9.64 (1.77)**</td>
</tr>
<tr>
<td><strong>LDL, mmol/L</strong></td>
<td>7.31 (1.86)</td>
<td>8.93 (1.82)§</td>
<td>9.54 (2.05)§</td>
<td>9.37 (1.52)§</td>
<td>7.78 (1.73)**</td>
</tr>
<tr>
<td><strong>TG, mmol/L</strong></td>
<td>1.65 (0.65-3.36)</td>
<td>1.38 (0.67-2.55)§</td>
<td>1.03 (0.4-2.55)§</td>
<td>1.63 (0.73-3.90)#</td>
<td>1.47 (0.67-2.09)</td>
</tr>
<tr>
<td><strong>HDL, mmol/L</strong></td>
<td>1.30 (0.42)</td>
<td>1.19 (0.28)</td>
<td>1.29 (0.31)</td>
<td>1.13 (0.22)</td>
<td>1.20 (0.36)</td>
</tr>
<tr>
<td><strong>Lp(a), mg/dL</strong></td>
<td>39.1 (3-105)</td>
<td>30.5 (5-93)</td>
<td>22.2 (4-60)</td>
<td>31.0 (5-93)</td>
<td>64.7 (20-102)</td>
</tr>
</tbody>
</table>

TC indicates total cholesterol; LDL, low-density lipoprotein cholesterol; TG, triglycerides; HDL, high-density lipoprotein cholesterol; and Lp(a), lipoprotein(a). All values are mean (±SD) except for TG and Lp(a) showing mean (95% confidence interval). LDL was calculated by Friedewald’s formula: LDL = TC − HDL − TG/2.2 mmol/L. For Lp(a), the number of individuals measured is shown in brackets.

*Untreated plasma lipid values were not available for all patients with familial hypercholesterolemia.

†Four individuals with gross deletions but unknown functions excluded from the subgroups of mutations.

§Includes 4 individuals with the E80K mutation, 2 with the S156L mutation, 3 with the P664L mutation, and 2 with gross deletions FH28 and FH218.

#P<.05 between defective protein in repeat 5 and null.

**P<.05 between defective protein in repeat 5 and defective protein excluding repeat 5.

in the 3' end of exon 4 of the LDL receptor gene. This region codes for repeat 5 of the binding domain of the LDL receptor, which we and others have shown is a frequent mutation site in FH patients. SSCP detected mutations in 29 of the 311 patients studied (9.3%). SSCP is known to be sensitive to the fragment size as well as the electrophoresis conditions, and we observed that a relatively small change in ambient temperature resulted in a variation in the ability to detect an SSCP. It was therefore important to control the experimental conditions for the SSCP run to achieve the most consistent outcome. Complete sequencing of this same DNA fragment from 30 FH patients who showed no SSCP changes did not reveal any additional mutations. We thus conclude that for this 236 bp fragment, single-condition SSCP has detected most of the mutations present in the sample.

In this group of FH patients, 9.3% had a mutation in the 3' end of exon 4, and this high frequency has also been reported from the Dallas collection of fibroblasts from homozygous FH patients, which represents patients from a wide geographic spectrum. The mutations are concentrated in a region of fewer than 50 bases, and to date, 13 mutations have been identified in this region, suggesting that a DNA sequence-specific mechanism may underlie the high frequency of mutations in this region of the gene (discussed in Reference 9). Another possibility for the high frequency is that patients with mutations in this region of the gene are more severely affected and are thus more frequently...

![Figure 3](http://atvb.ahajournals.org/)

**Fig 3.** Scatterplots show serum low-density lipoprotein (LDL) cholesterol levels against age in patients with a defective protein (a) \((r = .53, P = .001)\) and null alleles (b) \((r = .24, P = .4)\). The 95% confidence interval lines for the regression line are shown in panel a. Interactive outlier analysis showed that the offspring included in panel a do not alter the outcome on either plot.
found in lipid clinics. The data from our study support this idea and show that these patients have significantly higher pretreatment total and LDL cholesterol levels than the rest of the group, particularly compared with patients with missense mutations in other regions of the gene. Of the mutations detected in this study that result in a repeat 5-defective protein, both the del G197 and D200G have been shown to have less than 2% receptor activity\textsuperscript{8,11,22} and the D206E a 5% to 15% receptor activity\textsuperscript{23-27}. It thus appears that mutations in repeat 5 of the protein may be as deleterious as mutations causing null alleles, at least in regard to plasma lipid levels. However, it is of interest that in the patient group with a defective allele, cholesterol levels show an increase with age of diagnosis, as do the data from the entire patient group (not shown), whereas cholesterol levels are high at all ages in patients with a null allele. The explanation for this observation is unclear but is in agreement with results from other researchers.\textsuperscript{4}

In the general population a clear relation exists between the magnitude of hypercholesterolemia and the prevalence and incidence of coronary artery disease (CAD)\textsuperscript{28,31} as well as the onset of symptoms, with an increase of 1.3 mmol/L in plasma cholesterol associated with a CAD onset 10 years earlier. Thus, the 1.88 mmol/L higher total cholesterol in those individuals with missense mutations in repeat 5 and the 1.43 mmol/L higher levels in those with a null allele raise the possibility that these individuals would be more severely affected with CAD compared with the other FH patients. With the clinical information currently available for these patients, a definitive analysis of this could not be carried out at this time. In homozygous FH there is clear evidence that patients with lower levels of receptor activity have higher LDL cholesterol\textsuperscript{32} and a more rapid progress of atherosclerosis.\textsuperscript{1} Although a consistent correlation has not been demonstrated between serum cholesterol levels and the age of CAD onset in heterozygous FH individuals, there is some evidence to support the relation.\textsuperscript{23-36} In a large study of FH patients,\textsuperscript{33} men with CAD had significantly higher LDL cholesterol than those free of disease (7.13 versus 6.51 mmol/L), and in a recent study from South Africa,\textsuperscript{4} FH patients with CAD had 8% higher cholesterol than those without CAD. A significant positive correlation between intima-media thickening in the femoral artery and total serum cholesterol levels in FH patients has been reported.\textsuperscript{37} In a group of French Canadian heterozygous FH patients, individuals with the T66W mutation in exon 3 of the LDL receptor gene had lower plasma cholesterol levels (7.2 mmol/L) than individuals who were heterozygous for the French Canadian 10-kb deletion (8.0 mmol/L).\textsuperscript{3} Although there was no difference in the frequency of CAD between the individuals in the families, those who carried the deletion had CAD at an earlier age, supporting the view that the plasma level of cholesterol has an influence on CAD onset.

In the light of the observations in this report, as well as those by other researchers,\textsuperscript{2-5} it is now clear that different mutations are associated with differences in lipid levels, and it is likely that this will be associated with clinically different effects. It is also apparent that the phenotypic effect of the mutation is modulated by other genetic or environmental factors.\textsuperscript{4,38} Studies that identify the specific mutations in large groups of unrelated FH individuals of heterogeneous background such as have been described here will be important in defining the genotypic background on which the genetic and environmental effects on phenotype can be analyzed.

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