Six DNA polymorphisms in the low density lipoprotein receptor gene: their genetic relationship and an example of their use for identifying affected relatives of patients with familial hypercholesterolaemia

Steve Humphries, Linda King-Underwood, Wilmunder Gudnason, Mary Seed, Sophie Delattre, Veronique Clavey, Jean-Charles Fruchart

Abstract
We have determined the relative allele frequency and estimated linkage disequilibrium between six DNA polymorphisms of the low density lipoprotein (LDL) receptor gene. Polymorphisms were detected using the enzymes SfAlI, TaqI, Stul, HincII, AvaII, and NcoI after DNA amplification by the polymerase chain reaction. Strong linkage disequilibrium was detected between many of the pair wise comparisons in a sample of 60 patients heterozygous for familial hypercholesterolaemia (FH). Using the enzymes HincII, NcoI, and SfAlI, 85% of patients were heterozygous for at least one polymorphism and thus potentially informative for cosegregation studies. The polymorphisms were used to follow the inheritance of the defective allele of the LDL receptor gene in the relatives of a patient with FH. Assays of LDL receptor activity on lymphoblastoid cell lines from two members of the family was used to confirm that the proband, but not the hypercholesterolaemic brother, had a defect in the LDL receptor. In the family, none of the children had inherited the allele of the LDL receptor gene inferred to be defective. The problems associated with this cosegregation approach to identify relatives of patients with a clinical diagnosis of FH are discussed.

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Familial hypercholesterolaemia (FH) is a common inherited disease with a frequency of about 1 in 500. The disorder is characterised clinically by a raised concentration of low density lipoprotein (LDL) cholesterol in blood, tendon xanthomata, an increased risk of myocardial infarction, and genetically by autosomal dominant inheritance. Studies performed over 15 years ago in the UK and the USA indicate that 5 to 10% of subjects suffering a myocardial infarction before the age of 55 years may have FH. Hypercholesterolaemia is treatable both by dietary measures and by drug therapy, and it is therefore important to identify subjects who have FH so that appropriate treatment can be offered to reduce their subsequent risk of myocardial infarction.

FH results from different genetic defects in a cell surface receptor which normally controls the uptake of plasma LDL, and the cloning of the human LDL receptor gene has made it possible to study FH using DNA technology. There have been at least 40 different mutations of the LDL receptor gene reported, either gross structural alterations such as deletions or insertions, or those which arise from a single base pair change in the gene. Both of these types of alterations result in an LDL receptor with impaired function, thus causing FH. However, as well as allelic heterogeneity, genetic heterogeneity has recently been shown in FH patients. In studies in the UK and Germany, roughly 3% of patients with a clinical diagnosis of FH have a defect in the apolipoprotein (apo) B ligand for the receptor, with LDL receptor activity in these patients being within the normal range. The molecular defect underlying this disorder (familial defective apo B 100) is a single base change in the apo B gene causing a substitution of Arg3500 for Gln, in a region of the protein that interacts with the receptor. The defective binding and clearance of the LDL-Gln3500 causes the hypercholesterolaemia observed.

Although in many cases, diagnosis of FH can be carried out on the basis of a single total cholesterol estimation, there is general agreement that about 10 to 15% of children could not be diagnosed unequivocally by this means alone. This is because of overlap between plasma lipid values in normal and FH subjects, although this degree of overlap can be reduced somewhat by determination of LDL cholesterol. In addition, some children sampled before puberty may have cholesterol levels within the normal range for their age and gender and only develop raised cholesterol levels in later years. Diagnosis in the families of these patients may be greatly assisted by using cell biology or genetic tests. Several methods have been published to determine LDL receptor function in freshly isolated lymphocytes from patients. However, these methods are technically complex, and for all published methods there is not complete discrimination between subjects who have a proven defect in the LDL receptor and those who do not.

For such patients and their relatives, genetic tests would be a useful way to alleviate dia-
gnostic conundrums. In the UK, detectable gross deletions in the LDL receptor gene are found only in about 5% of patients, and to date seven different point mutations or small deletions have also been reported. Some of these occur in several apparently unrelated subjects and, taken together, these mutations and deletions accounted for approximately 20% of the molecular defects in a group of 200 FH patients from London. However, for the remainder of patients, restriction fragment length polymorphisms (RFLPs) of the LDL receptor gene can be used to follow the inheritance of the defective gene in the family, and to identify relatives who have inherited the defective allele.

We report here the use of the polymerase chain reaction (PCR) gene amplification technique to determine the genotype at six variable sites in the LDL receptor gene. We have estimated the extent of linkage disequilibrium between the variable sites, and have used a combination of cellular and genetic techniques to study a family from London, and to identify those subjects who have inherited the defective LDL receptor gene.

**Methods**

**SELECTION OF SUBJECTS**

Patients with a clinical diagnosis of FH were recruited from lipid clinics in London. The diagnostic criteria used were as previously described, and, briefly, comprised an increased level of total and LDL cholesterol in plasma (over the 95th centile for the general adult (>18 years) population (>4.9 mmol/l)) with a total triglyceride of not more than 2.0 mmol/l, together with tendon xanthomata or premature coronary artery disease in the patient or in a first degree relative.

**DETECTION OF THE LDL RECEPTOR RFLPS**

Genomic DNA was isolated from venous blood as previously described. Fragments of the LDL receptor gene were amplified using the polymerase chain reaction with the oligonucleotides (Oswell DNA Services, Department of Chemistry, University of Edinburgh, or Severn Biotech Ltd) shown in table 1. The reaction mix contained approximately 500 ng of genomic DNA, 150 to 250 ng of each oligonucleotide (table 1), 200 mmol/l of each dNTP (BRL), 10% (v/v) dimethyl sulphoxide, and 0.4 U of Taq polymerase, in a total volume of 25 µl of buffer as recommended by the manufacturer (Perkin-Elmer Cetus, USA). The optimum concentration of magnesium chloride was determined for each oligonucleotide pair, and was between 1.0 and 1.5 mmol/l. Thirty cycles of amplification were carried out in a programmable heating block (Cambio, Cambridge, UK) with denaturing at 95°C for one minute, annealing at 55°C for one minute (62°C for the AvaII oligonucleotides), and extension for one minute at 72°C. One tenth of the product was analysed by agarose gel electrophoresis (Ultrapure Agarose, BRL) and visualised with ethidium bromide. For the SfaNI variable site in exon 2 the polymorphism was detected by differential oligonucleotide hybridisation using allele specific oligonucleotides and conditions as described previously.

**ASSAY OF LDL RECEPTORS ON LYMPHOBLASTOID CELLS**

Human mononuclear cells were isolated under sterile conditions from fresh blood of a normal donor (MF), a heterozygous familial hypercholesterolaemic patient (4A, deletion in exons 9 and 10 of the LDL receptor gene), and from the proband in family Y (II.1) and his brother (II.3) according to the method of Boyum. The cells were transformed with Epstein-Barr virus as described by Neitzel and cultured in RPMI-1640 medium (Gibco), penicillin (Seromed) 100 units/ml, streptomycin (Seromed) 100 µg/ml, and fetal calf serum (Boehringer) 10% v/v. Twenty-four hours

<table>
<thead>
<tr>
<th>Primer (5'-3')</th>
<th>Location</th>
<th>Fragment size (bp)</th>
<th>Enzyme for RFLP</th>
</tr>
</thead>
<tbody>
<tr>
<td>CTCTGCCCTTCTTCTCTCTCCTCAGAAAATAATGCAATACTGCGAAA</td>
<td>Intron 1</td>
<td>1</td>
<td>913</td>
</tr>
<tr>
<td>ATCTGACGGGAAAACTGGCG</td>
<td>End exon (Nt 675)</td>
<td>4</td>
<td>978</td>
</tr>
<tr>
<td>GGCCAGCTGGAAAACGGA</td>
<td>End intron 4</td>
<td>1090</td>
<td>150</td>
</tr>
<tr>
<td>ATCGATGCTGGCTGATCCTCPTCCTCCTGTTCTGAAGC</td>
<td>Start exon (Nt 1062)</td>
<td>8</td>
<td>150</td>
</tr>
<tr>
<td>CCTGTCATGCCTCTCCCTGTCCTCG</td>
<td>End exon 8</td>
<td>Start exon 12</td>
<td>180</td>
</tr>
<tr>
<td>TGGCTGGCTTGATGCTGATGCAAACGTTG</td>
<td>End exon 12</td>
<td>180</td>
<td>130 + 50</td>
</tr>
<tr>
<td>TGGCTGGCTTGATGCTGATGCAAACGTTG</td>
<td>Start exon 13</td>
<td>157</td>
<td>128 + 29</td>
</tr>
<tr>
<td>CACCTGACTCCCTGACTGGGTAAG</td>
<td>End exon 13</td>
<td>916</td>
<td>430 + 485</td>
</tr>
</tbody>
</table>

* Detected in this study by ASO. † 1% agarose. ‡ 2% NuSieve/2% agarose.
before the tests, cells were washed with RPMI and preincubated in RPMI-1640 medium supplemented with bovine serum albumin (BSA from IBF) 10 g/l replacing FCS, to a final concentration of 10^6 cells/ml, to enhance the LDL receptor expression on the cell surface.

Human LDL (density 1.030 to 1.055 g/ml) was isolated from fresh plasma of normal healthy donors by four sequential centrifugation steps^42 at 100,000 g in a 50-2 TI Rotor in a Beckman L-8 ultracentrifuge at 4°C for 24 hours. LDL was radiolabelled with [32P]Na by the iodine monochloride method described by MacFarlane^43 and modified by Bilheimer et al^44 for lipoproteins.

Analysis of the LDL receptor activity was as previously described. Briefly, the cells were resuspended in the preincubation medium and aliquots of 1 ml (10^6 cells) were prepared in 1.5 ml microfuge tubes. Cells were centrifuged at 1000 g for five minutes at 20°C in a Jouan CR412 centrifuge. Cells were incubated in multi-well plates at 4°C with five different concentrations of [32P]-LDL (2, 5, 10, 25, and 50 pg/ml) in triplicate with 10^6 cells in a final volume of 250 μl of RPMI + BSA. Non-specific binding was determined in duplicate by adding a 20 fold excess of unlabelled LDL. Cells were incubated at 4°C for two hours, the plate was centrifuged for five minutes at 1500 rpm, and the supernatant discarded. The cells were washed twice by centrifugation for five minutes at 1500 rpm with Buffer C (PBS, 0.01 mol/l, CaCl2, 0.5 mmol/l, BSA 2% p/v). The cell pellet was resuspended in 250 μl PBS 0.01 mol/l and transferred to tubes, followed by centrifugation for five minutes at 4000 rpm. The cell pellet was dissolved in 250 μl of 0.1 mol/l NaOH, the cell associated radioactivity was determined, and an aliquot of 100 μl was used for protein determination.

Binding internalisation and degradation of [32P]-LDL at 37°C was determined after four hours at five different concentrations of [32P]-LDL, as described above, by the procedure of Goldstein and Brown. The cells were washed and treated as previously described for the binding at 4°C.

**STATISTICAL ANALYSIS**

χ² analysis was used to test the hypothesis of random association between the alleles of two particular RFLPs. Statistical significance was considered to be at the 0.05 level. The correlation coefficient Δ was used to determine the degree of linkage disequilibrium between two RFLP loci A and B. Pairwise polymorphism information content (PIC) values were calculated from the observed frequency of the haplotypes in the control population. For subjects heterozygous for both polymorphisms, the relative frequency of the two possible haplotypes was estimated from the frequency of the unambiguously observed haplotypes, using an estimation maximisation algorithm.

**Results**

The position in the LDL receptor gene of the six variable sites used in this study is shown in fig 1. They are located throughout the gene, spanning a total distance of 35 kb from the most 5′ (SfaNI, exon 2) to the most 3′ (NcoI, exon 18). Five of these variable sites are in exons: SfaNI (exon 2), Stul (exon 6), HincII (exon 12), AaII (exon 13), and NcoI (exon 18 in the 3′ untranslated region of the mRNA), and only the StuI sites result in an amino acid change in the protein (Ala219→Thr). None of the variable sites affects any known functional sequence of the LDL receptor.

The oligonucleotides used to amplify the fragments of the LDL receptor gene are shown in table 1. For the SfaNI variable site in exon 2 the resulting fragments are small, and it is more convenient to detect the polymorphism by allele specific hybridisation. Table 2 shows the relative rare allele frequencies for the RFLPs in a sample of 60 unrelated FH patients from London. For all RFLPs, the observed genotype distribution was that expected for a population in Hardy-Weinberg equilibrium (not shown). The frequencies range from 0.03 (StuI) to approaching 0.5 (AaII, HincII). However, as shown in table 3, there is strong linkage disequilibrium between many of the pairwise combinations of RFLPs. By inspection of table 2, it would appear that the combination of HincII, NcoI, and SfaNI would be most informative, and in this sample of 60 patients a total of 51 were heterozygous for one or more of these three polymorphisms, (cumulative frequency 62%, 78%, and 85% respectively, not shown). No increase in the number of heterozygotes was observed from the use of the StuI RFLP and only one additional subject was heterozygous for the TaqI RFLP.

These RFLPs were used to follow the inheritance of alleles of the LDL receptor gene in the Y family (fig 2). The proband (II-1, table 3) has a clinical diagnosis of FH, with hypercholesterolaemia, coronary artery disease (angina at 35 years), and several first degree relatives with either hypercholesterolaemia or
CAD (table 3). He originally approached us with a request for diagnosis for his two sons, both of whom have raised total and LDL cholesterol levels for their age and gender (>75th centile, III.1, >95th centile III.2) as do the sons of his sister, II.5. Genotypes for several RFLPs were determined and the results for NcoI and TaqI are shown in fig 2. The affected mother of the proband is heterozygous for the NcoI RFLP and the N- allele has been inherited by the proband and his hypercholesterolaemic sister (II.5) but not by his hypercholesterolaemic brother (II.3). There is thus no correspondence between the inheritance of hyperlipidaemia and a particular allele of the LDL receptor and this raised the possibility that the hypercholesterolaemia in one or more of these subjects was not the result of a defect in the LDL receptor. None of these subjects was a carrier of the apo B Arg3500→Gln mutation (not shown), and LDL from the proband and his brother bound with normal affinity to LDL receptors in a U937 growth assay (J Frostedgard, J Nielsen, A Hamsten, unpublished data). Using RFLPs of the apo B gene, cosegregation could not be shown between alleles of the apo B gene and the hyperlipidaemia (not shown). Inspection of the LDL receptor and apo B RFLP data showed no evidence of biological inconsistency.

To examine this further, lymphoblastoid cell lines were established from the proband and his brother. LDL receptor function was compared to that of a cell line from a normal subject (MF) and from an FH patient from Iceland (4A), who was heterozygous for a deletion of exon 9–10 of the LDL receptor gene, and thus served as an unambiguous LDL receptor defective control. Assays were carried out to determine binding of LDL to the four cell lines at 37°C and 4°C using [125I]-LDL. The results from all assays were consistent and those from the 4°C binding are shown in fig 3. Binding to the cells from the proband was consistently low and similar to that of the FH cell line. Binding to the cells from the brother (II.3) was consistently high and similar to that seen for the normal cell line (MF). This suggests that the proband is heterozygous for a defect in the LDL receptor, while in his brother, LDL receptor function is within the normal range.

By inspection of the pedigree, the defective allele of the LDL receptor gene that has been inherited from the mother by the proband and his sister (but not by the brother) appears to

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**Table 3** Characteristics, age (at sampling in 1989), lipid levels, and NcoI and TaqI genotypes of members of the Y family.

<table>
<thead>
<tr>
<th>Pedigree No</th>
<th>Gender</th>
<th>Age</th>
<th>Total chol (mmol/l)</th>
<th>Trig (mmol/l)</th>
<th>LDL chol (mmol/l)</th>
<th>Genotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>M</td>
<td>73</td>
<td>7.8±</td>
<td>1.3</td>
<td>-</td>
<td>++/-</td>
</tr>
<tr>
<td>I.2</td>
<td>F</td>
<td>65</td>
<td>7.8±</td>
<td>1.7</td>
<td>5.7</td>
<td>-/++</td>
</tr>
<tr>
<td>II.1</td>
<td>M</td>
<td>41</td>
<td>8.3±</td>
<td>1.0</td>
<td>6.0</td>
<td>+/+</td>
</tr>
<tr>
<td>II.2</td>
<td>F</td>
<td>39</td>
<td>4.4</td>
<td>0.8</td>
<td>-</td>
<td>++/-</td>
</tr>
<tr>
<td>II.3</td>
<td>M</td>
<td>39</td>
<td>9.1±</td>
<td>1.4</td>
<td>7.0</td>
<td>-/+</td>
</tr>
<tr>
<td>II.4</td>
<td>F</td>
<td>42</td>
<td>6.7</td>
<td>0.5</td>
<td>5.0</td>
<td>+/+</td>
</tr>
<tr>
<td>II.5</td>
<td>F</td>
<td>36</td>
<td>8.7±</td>
<td>0.7</td>
<td>6.9</td>
<td>+/+</td>
</tr>
<tr>
<td>II.6</td>
<td>M</td>
<td>34</td>
<td>5.6</td>
<td>1.0</td>
<td>3.7</td>
<td>+/+</td>
</tr>
<tr>
<td>III.1</td>
<td>M</td>
<td>7</td>
<td>4.8</td>
<td>0.4</td>
<td>2.9</td>
<td>+/+</td>
</tr>
<tr>
<td>III.2</td>
<td>M</td>
<td>3</td>
<td>5.4</td>
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<td>+/+</td>
</tr>
<tr>
<td>III.3</td>
<td>M</td>
<td>8</td>
<td>4.9</td>
<td>0.4</td>
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<td>III.5</td>
<td>M</td>
<td>2</td>
<td>5.7±</td>
<td>1.3</td>
<td>3.8</td>
<td>+/+</td>
</tr>
</tbody>
</table>

* = Cholesterol over 95th centile for age and gender

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**Figure 2** Pedigree of the Y family. Cholesterol levels (mmol/l) in the family members. Subjects with cholesterol levels over the 95th centile for their age and gender are indicated by *. Genotypes for the NcoI and TaqI RFLPs are shown. The proband is indicated with an arrow.
cosegregate with the N- allele of the NcoI RFLP. Of the children, only III.1 has inherited the N- allele, and may therefore have inherited the defective allele of the LDL receptor. However, this N- allele may have been inherited from the mother (II.2), and to examine this possibility other RFLPs were used. Both HincII and TaqI were informative in this family and TaqI genotypes are presented. By inspection, the defective allele in this family segregates with a T- allele, which has not been inherited by III.1. We thus deduce that none of the four children has inherited the defective LDL receptor allele inferred to be present on the T-N haplotype in the grandmother.

Discussion

There are several potential problems in diagnosing FH in the children of an FH heterozygote. Few lipid laboratories have established normal ranges of lipid levels for children, although several cross sectional studies have reported that the mean and 95th centile of lipid levels in children are significantly lower than for adults. Thus, a child who is heterozygous for FH and has a total or LDL cholesterol level greater than the 95th centile for age and gender may have levels below the 95th centile for an adult and may not be correctly identified. This has been confirmed by a recent study from Finland where relatives of patients with a known mutation in the LDL receptor gene were investigated. Based on unequivocal DNA diagnosis, 15% of the relatives were misclassified using only adult lipid data to determine hypercholesterolaemia (lipid levels above the 95th centile). This misclassification was reduced using age and gender specific lipid values, but there were still 5% of relatives who were incorrectly classified as 'FH' or 'normal' by lipid values alone.

Until recently, diagnosis of FH in children has been in question only in the 10 to 15% of subjects whose total or LDL cholesterol levels fall within the 'overlap' of the bimodal distributions observed in first degree relatives of subjects with FH. However, a recent longitudinal study indicated that there are children at risk for FH in whom total or LDL cholesterol levels may initially be well within the normal range, and may subsequently rise to levels at which diagnosis of FH would have been made unequivocally using conventional criteria. Both of these problems can be overcome by the application of genetic tests. In patients in the UK, a number of gross deletions, small deletions, and point mutations have been reported, explaining in total only 20% of the defects in 200 patients studied in London, and thus in the majority of patients the molecular defect is still unknown.

The main aim of this study was therefore to determine the usefulness of six RFLPs of the LDL receptor gene as cosegregation markers for diagnosis in families with known FH.

The usefulness of such polymorphisms depends on the relative frequency of the alleles and the degree of linkage disequilibrium between the alleles of the different polymorphisms. We have previously reported similar studies using Southern blotting methods and the enzymes NcoI, ApalI, PvuII, and StuI, showing an overall heterozygosity index of 0.07 with a combination of AcoII and NcoI being the most useful pair (PIC 0.61). However, the ApalI and PvuII variable sites are located in the 5 kb long intron 15, and we have been unable to amplify this intron using primers for exon 15 and 16 (unpublished data). For the six RFLPs used in the current study, the combination of HincII and NcoI gives the highest pairwise PIC value of 0.68, and with the addition of the SfaNI RFLP, 85% of patients in the sample were heterozygous for at least one polymorphism. Although the SfaNI RFLP is of low frequency (rare allele frequency 0.14), the extent of the linkage disequilibrium with the other polymorphism is also low, with the presence of the (rare) SfaNI ‘no cutting’ site being found most frequently on the haplotype containing the common allele for the HincII and NcoI RFLPs (not shown). As expected from our previous studies, the low frequency of the StuI variable site (reported as 0.06 to 0.03) means that, in general, it is of little use in family investigations. Recently, variable copy dinucleotide repeat polymorphisms have been reported in the LDL receptor gene. Although technically more difficult than the PCR methods we present here, these polymorphisms should also be useful for family studies.

The investigation in the Y family illustrates a number of the problems that may be encountered in attempting to identify familial hypercholesterolaemia in the relatives of a patient with a clinical diagnosis of FH. With an estimated population carrier frequency of defects causing FH of 1/500, families where both parents have LDL receptor defects are rare.
However, there are many other genetic and environmental factors that cause hyperlipidaemia, and familial aggregation of these environmental factors and segregation of other undetected genetic variants may complicate the unequivocal identification of the defective LDL receptor allele. This appears to be the case in family Y, where it may be that a mutation in a gene (or combination of genes) causing hyperlipidaemia has been inherited (possibly from the grandfather (I.1)) by several of the family members (II.3, II.1.2, and III.6). The use of the LDL binding assay allowed the inheritance of the defective allele to be inferred in this family. Although the cosegregation analysis showed that none of the four children in generation III had inherited the defective allele of the LDL receptor from the grandmother, it leaves unresolved the reason for the hypercholesterolaemia seen in II.3 and II.6.

With the availability of many RFLPs of the LDL receptor gene, the main factors determining the success of diagnosis by a family cosegregation approach are uncertainty in the diagnosis, caused by individual differences in genetic background or environmental factors, unavailability (owing to premature death) of affected relatives to determine phase, and the confounding problem of non-paternity. In situations of doubt, paternity can be confirmed using genetic tests, and cosegregation study of families of patients with FH from Munich has shown that diagnosis was possible in all cases where samples from four or more relatives were available, though this depends on the pedigree structure. In the Y family the analysis required the determination of LDL receptor function in the proband and his hypercholesterolaemic brother. This shows the usefulness of the cell LDL receptor assay and the complementary nature of the cellular and genetic tests. To date, no cell assay of LDL receptor function allows an unambiguous distinction between cells from normal and LDL receptor defective subjects. In the next few years, it is increasingly likely that the problems of diagnosis by family studies will be avoided by the identification of specific mutations in the LDL receptor gene. Rapid methods such as SSCP for identifying new mutations in individual patients will be useful in the future, but until these become routine, unequivocal identification of carriers and non-carriers in the relatives of FH patients may be aided by cosegregation studies using the RFLPs described here.

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Six DNA polymorphisms in the low density lipoprotein receptor gene


