Familial hypercholesterolemia (FH) and familial defective apolipoprotein B-100 (FDB) represent ligand-receptor disorders that are complementary. Individuals with both FH and FDB are unusual. We report a family with both disorders and the impact of the mutations on the phenotypes of the family members.

Methods: We used single strand conformation polymorphism (SSCP) and denaturing gradient gel electrophoresis (DGGE) for genetic analysis of all 18 exons and the promoter region of the LDL receptor and DGGE for genetic analysis of the apolipoprotein B-100 (apo B-100) gene. The functional significance of the apo B-100 mutation was studied using a U937 cell proliferation assay. Fasting serum lipid profiles were determined for the index case and seven first-degree relatives.

Results: One of the patient’s sisters had a missense mutation (Asp407→Lys) in exon 9 of the LDL receptor and a serum LDL-cholesterol concentration of 4.07 mmol/L. Four other first-degree relatives had hyperlipidemia but no LDL-receptor mutation. However, these subjects had a mutation of the apo B-100 gene (Arg3500→Trp). The cell proliferation rate of U937 cells fed with LDL from other subjects with the same mutation was fourfold less than that of controls. The index case had both FH- and FDB-related mutations. Her serum LDL-cholesterol (9.47 mmol/L) was higher than all other relatives tested.

Conclusions: Existence of both FH and FDB should be considered in families with LDL-receptor mutations in some but not all individuals with hypercholesterolemia or when some individuals in families with FH exhibit exaggerated hypercholesterolemia.

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Familial hypercholesterolemia (FH),

4 an autosomally dominant inherited defect of LDL receptors, produces premature coronary artery disease (CAD) and occurs in 1 in 500 individuals (1, 2). Familial defective apolipoprotein B-100 (FDB) results from a mutation at the apolipoprotein B-100 (apo B-100) locus on chromosome 2, leading to defective binding of LDL to the LDL receptor (3). It also occurs in ~1 in 500 individuals and leads to premature CAD. FDB cannot be differentiated from FH phenotypically (4). Subjects with both FH and FDB are rare, and there have been only three reports in the literature of such compound heterozygotes (5–7).

Here we describe a proband who was identified to have both FH and FDB, detected as part of a family screening program for the detection of FH.

Materials and Methods

The proband was identified based on the presence of severe hypercholesterolemia and the presence of tendon xanthomata in accordance with established diagnostic criteria (8). Seven first-degree relatives of the proband,

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4 Nonstandard abbreviations: FH, familial hypercholesterolemia; CAD, coronary artery disease; FDB, familial defective apolipoprotein B-100; apo, apolipoprotein; LDL-C, LDL-cholesterol; SSCP, single strand conformation polymorphism; and DGGE, denaturing gradient gel electrophoresis.
ages 14–63 years, were studied. All were free of symp-
toms of CAD and had normal resting electrocardiograms.
Nephrotic syndrome and hypothyroidism were excluded.
This study was approved by the ethics committee of the
Singapore General Hospital. Informed consent was ob-
tained from all subjects studied.

Blood was taken after a 10-h fast. Cholesterol and
triglyceride concentrations were measured by enzymatic
methods using Kodak Ektachem chemistry slides, which
were then read on a Vitros 700 Chemistry Analyzer.
HDL-cholesterol was measured after precipitation with
dextran sulfate and magnesium chloride. LDL-cholesterol
(LDL-C) was calculated using Friedewald’s formula (9).
Only the pretreatment lipid profile is presented.

DNA was extracted from whole blood using phenol-
chloroform extraction and salt precipitation.

Mutations in the LDL-receptor gene were detected
independently using PCR-single strand conformation
polymorphism (SSCP) analysis and denaturing gradient
gel electrophoresis (DGGE) in two laboratories.

PCR-SSCP

Each PCR was performed in a final volume of 50 µL
containing 0.2 µg of genomic DNA, 25 pmol of each
oligonucleotide primer, 200 µmol of deoxynucleotide
triposphates, and 1 U of Taq DNA polymerase in the
reaction buffer supplied (Promega). PCR was carried out
using the GeneAmp 9700 PCR system (Perkin-Elmer). The
oligonucleotide primers for the promoter region and the
translated exon sequences were as described previously
(10). The PCR and SSCP conditions are shown in Table 1.
After amplification, 5 µL of the PCR product was added
to 5 µL of loading buffer (950 mL/L formamide, 0.5 g/L bromphenol blue, 50 g/L EDTA), denatured by heating at
95 °C for 5 min, and quenched on ice. SSCP analysis was
carried out using the DCode Universal Mutation Detection
System (Bio-Rad). Samples were electrophoresed in
acrylamide gel without glycerol in Tris-borate-EDTA (89
mmol/L Tris base, pH 8.3, 89 mmol/L boric acid, 2
mmol/L disodium EDTA) at room temperature for 18–20 h at 120 V. The gel was stained with silver nitrate (Silver Sequence DNA Sequencing System; Promega).

Table 1. PCR and SSCP conditions for analysis of the LDL receptor.

<table>
<thead>
<tr>
<th>Amplified segment</th>
<th>Denaturation, annealing, extension temperature, °C (time) for PCR</th>
<th>SSCP conditions</th>
</tr>
</thead>
<tbody>
<tr>
<td>Promoter</td>
<td>95 (30 s), 63 (1 min), 72 (1 min)</td>
<td>Acrylamide:bis-acrylamide ratio</td>
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<tr>
<td>2</td>
<td>95 (30 s), 55 (1 min), 72 (1 min)</td>
<td>39:1</td>
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<td>39:1</td>
</tr>
<tr>
<td>4A</td>
<td>95 (30 s), 66 (1 min), 72 (1 min)</td>
<td>37.5:1</td>
</tr>
<tr>
<td>4B</td>
<td>95 (30 s), 68 (1 min), 72 (1 min)</td>
<td>39:1</td>
</tr>
<tr>
<td>5</td>
<td>95 (30 s), 58 (1 min), 72 (1 min)</td>
<td>37.5:1</td>
</tr>
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<td>18</td>
<td>95 (30 s), 60 (1 min), 72 (1 min)</td>
<td>39:1</td>
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</table>

* All thermal cycling consisted of 35 cycles and was completed by thermal extension for 10 min at 74 °C.
denaturation step at 95 °C for 5 min, 40 cycles at 94 °C for 1 min and 66 °C for 5 min, and a final extension step at 72 °C for 10 min. This was then followed by a denaturation/renaturation program at 99 °C for 7 min, 65 °C for 1 h, and 37 °C for 1 h, with a final soak at 4 °C, to generate hetero- and/or homoduplexes from the amplified PCR products.

Parallel denaturing gradient gels of 20–60%, 30–70%, or 40–80% denaturant (100% denaturant, 7 mol/L urea plus 400 mL/L formamide) were made with a gradient gel mixer. In brief, amplified PCR products from the apo B-100 gene were loaded onto 20–60% gels; PCR products from exons 2, 3, 5, 6, 10, 11, 12, 13, and 17 and the promoter region (LDL-receptor gene) were loaded onto 30–70% gels; and those from the remaining LDL-receptor exons (1, 4–5, 4–3, 7, 8, 9, 14, 15, 16, and 18) were loaded onto 40–80% gels and then electrophoresed for 6 h at 150 V.

Analysis of the apo B-100 gene used DGGE as described previously (12). The primers used were 5'-CGCCCGCGCGCCCGCGCCCGTCCCGCCGCCCCGCCC-CCGGAGGACTTGGACCAAGCTTATTGC-3' with a 40-bp GC-clamp at the 5' end, and 5'-GGTGCGTTTCTGGCTTGT-ATGTTCTCC-3'. The putative LDL-receptor binding region containing nucleotides 10551–10892 (corresponding to codons 3448–3562) of exon 26 of the apo B-100 gene was amplified and screened for mutations.

All mutations found by SSCP or DGGE were confirmed by DNA sequencing. For the LDL receptor, we used the ABI Prism 310 Genetic Analyzer (Perkin-Elmer). Genomic DNA was amplified using the primers for the exon in which SSCP or DGGE revealed a mutation. PCR conditions were in accordance with the manufacturer’s recommendation. The product was cleaned with QIAquick PCR purification reagent set (Qiagen) before sequencing. The apo B-100 gene was sequenced using the same primers without the GC-clamp as described previously (12). apo E was genotyped by the method of Hixson and Vernier (13).

LDL from the patients in this family was not available for studies to assess the functional significance of the apo B-100 mutation found. However, we were able to obtain material from 8 other patients with the same mutation, and the functional significance of the mutation was assessed by comparing the results of a U937 cell proliferation assay (14, 15) in these 8 subjects against those of 10 healthy controls. Because Van den Broek et al. (15) found that the cell density at the beginning of the experiment influenced U937 cell proliferation, standardized conditions were strictly followed in this study: (a) A fixed number of cells (1 × 10^5 cells/mL) was used in all experiments. (b) The cells were incubated in medium without serum for 24 h to ensure intracellular cholesterol depletion. (c) Frostegard et al. (14) observed that oxidized LDL reduced the ability to stimulate U937 cell proliferation. Because naturally occurring antioxidants in the d >1.21 kg/L fraction may prevent oxidation of the LDL, only pure LDL was used for the experiments.

Results

The pedigree for the family is shown in Fig. 1. A mutation in exon 9 of the LDL receptor was detected in the proband (subject II-4) and one sibling (subject II-1) by both PCR-SSCP (Fig. 2) and PCR-DGGE analyses. Subsequent sequencing showed that this was a C-to-G substitution at nucleotide position 1284 (Asp407→Lys), a mutation that has previously been shown to cause FH in South Africans (16). In addition, a polymorphism in exon 10 (G-to-A substitution at nucleotide 1413) was detected in both the proband and her sister (subject II-1). This has been found to be common in healthy individuals (17). No other abnormalities in the LDL-receptor gene were detected. Clinical and biochemical characteristics of the family

![Fig. 2. SSCP of exon 9 of the LDL receptor for the family members studied demonstrating the abnormal pattern caused by a mutation in exon 9 (Asp407→Lys). Subjects are the same as in Fig. 1.](image-url)
members are shown in Table 2. The father of the proband (subject I-2) suffered a sudden death at the age of 45 years (20 years before the diagnosis of FH in the proband), and no additional information was available for him. Several members of the family (subjects I-1, II-3, II-4, II-5, III-1, and III-2) had increased serum cholesterol (>7.5 mmol/L), which is diagnostic of FH in first-degree relatives of a proband (8), but did not carry the mutation. DGGE of the apo B-100 gene (Fig. 3) showed that all but one of these subjects, i.e., the mother of the proband (subject I-1), a brother (subject II-5), and two sons (subjects III-1 and III-2) as well as the proband herself (subject II-4), had the same mutation in the apo B-100 gene (Arg3500→Trp). The proband was thus a compound heterozygote for both FH and FDB. All members of this family had the ε3ε3 wild-type genotype for apo E, which is not associated with hypercholesterolemia. The hypercholesterolemia in subject II-3 is currently unexplained. To rule out the possibility of an LDL-receptor mutation that may have been present and undetected by SSCP or DGGE, we sequenced all exons and the promoter region in this subject. No additional abnormalities were detected.

The results of the U937 cell proliferation assay are shown in Fig. 4. The cell proliferation rate at 20 μmol/L LDL-C (mid-point of this assay) was 0.77 for cells fed with LDL from the FDB subjects with the Arg3500→Trp mutation, compared with 4.01 for cells fed with LDL from healthy individuals, a fourfold reduction in the cell proliferation rate. This trend was similar to those seen by Gaffney et al. (18) and Van den Broek et al. (15) with LDL from patients with the classical Arg3500→Gln mutation.
Discussion

Exons 7–14 of the LDL-receptor gene encode the epidermal growth factor precursor homology domain of the LDL-receptor protein, consisting of ~400 amino acids (19, 20). This domain is required for the acid-dependent dissociation of the receptor from its ligand during recycling (21). Thus, the amino acid substitution, from asparagine to a positively charged lysine in exon 9, found in some of our study subjects might interfere with receptor recycling. The structure and function of this mutation have yet to be studied.

When it was first described, FDB was thought to be produced by a single mutation at codon 3500 of the apo B-100 gene (22, 23). Since then, several other mutations have been found, some of which were first described in an Asian population (12). It was one of these mutations (Arg3500→Trp) that was found in this family. We have now shown that this mutation leads to a loss of function similar to that observed with the classical Arg3500→Gln mutation (Fig. 4).

FDB represents a ligand-receptor disorder that is complementary to FH. Compound heterozygotes with both FDB and FH are unusual, with only three reports of compound heterozygotes in the literature (5–7). Of these, only two groups characterized the mutations by DNA analysis (6, 7). They found, as we did, that compound heterozygotes exhibited more severe hypercholesterolemia than did their first-degree relatives heterozygous for either FH or FDB. The proband in this family had a pretreatment serum LDL-C concentration almost twice as high as those of her siblings (Table 2). Such compound heterozygotes, we believe, may account for two other phenomena occasionally observed in FH kindred. (a) Families have been reported in which an LDL-receptor defect was not found in all of the hypercholesterolemic first-degree relatives of patients with FH (24). This was the case in this family, and only DNA analysis of the apo B-100 gene allowed an accurate diagnosis to be reached. The presence of a compound heterozygote in the family must be considered in such cases and apo B-100 mutations sought actively to avoid labeling an affected individual as healthy, especially in children in whom the lipid profiles are less remarkable, such as subjects III-1 and III-2. It is also necessary to look for mutations other than the original Arg3500→Gln mutation because several other mutations are known to cause FDB in Asians. (b) Some FH kindreds, like the one presented here, include individuals with exaggerated hypercholesterolemia compared with other family members who have heterozygous FH. We should consider the possibility that these individuals are compound heterozygotes for FH and FDB.

Although the Asp407→Lys mutation found in exon 9 of the LDL receptor in the proband and in subject II-1 has been reported previously as a cause of FH (16), we have not demonstrated functional impairment of the LDL receptor in these individuals. The unexplained hypercholesterolemia seen in subject II-3 (Table 2) raises the possibility that another, currently unidentified mutation may contribute to the hypercholesterolemia seen in the proband as well as in subjects II-1 and II-3. However, no other mutations were found in the LDL receptors of our patients even with direct sequencing of the entire coding region, and we believe that the exon 9 mutation we found is significant. Of course, we cannot rule out the possibility that genes at another locus could interact with mutations at the LDL receptor or apo B-100 locus and may produce variations in phenotype in affected family members. In the first reported family of this type, the compound heterozygote was found to have lipid concentrations similar to the concentrations in individuals with either defect (5). The authors attributed this to other genetic or environmental factors that may have been operating in this individual. Another example of such gene-gene interaction is seen the recent work of Knoblauch et al. (25), in which a locus on chromosome 13q was linked to variations in cholesterol concentrations in a family with FH. Although we agree that this deserves further investigation, we are unable to speculate any further at this time.

Despite the exaggerated hypercholesterolemia, FH/FDB compound heterozygotes retain an ability to respond to lipid-lowering therapy (7). In our proband, treatment with 80 mg/day simvastatin lowered LDL-C from 9.47 mmol/L to 4.82 mmol/L. This represents a 49% reduction, which is similar to the 46% and 48% reductions in total cholesterol seen in the two cases reported by Benlian et al. (7).

When one genetic defect fails to explain the cause of hypercholesterolemia in families who appear to have monogenic hypercholesterolemia, the presence of both LDL receptor and apo B-100 defects should be considered.

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References


hypercholesterolemia and familial defective apolipoprotein B-100.