

Screening for mutations in exon 4 of the LDL receptor gene: identification of a new deletion mutation

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Abstract

DNA from 14 unrelated New Zealand familial hypercholesterolaemia (FH) heterozygotes, originating from the United Kingdom, was screened for mutations in exon 4 of the low density lipoprotein receptor (LDLR) gene. One patient was heterozygous for mutation D206E, which was initially identified in South Africa. The chromosomal background of this mutant allele was compatible with that described previously in Afrikaner and English patients, suggesting that this mutation originated in the United Kingdom. The 2 bp deletion in codon 206 and mutations D154N and D200G, previously reported in English FH patients, were not detected in this sample. In one of the patients, however, a new deletion of 7 bp was identified after nucleotide 581 (or 582) in exon 4 of the LDLR gene.

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Familial hypercholesterolaemia (FH) is caused by mutations in the low density lipoprotein receptor (LDLR) gene.¹ Variation in the clinical expression of heterozygous FH often complicates the diagnosis of this autosomal dominant disease, which is characterised by raised LDL cholesterol levels and the presence of tendon xanthomata and coronary heart disease (CHD). Although accurate diagnosis of FH is possible by means of molecular methods, their use in heterogeneous populations is limited at present owing to mutational heterogeneity of the LDLR gene. In some parts of the world, however, where specific FH related mutations occur in a large proportion of patients, owing to "founder effects" resulting from migration and cultural or geographical isolation,²⁻⁶ cost effective DNA diagnosis of FH can be applied. We have previously reported on the value of a molecular diagnostic service for FH in Afrikaners⁷ where three founder related LDLR gene mutations are responsible for FH in up to 90% of cases.⁵ Screening for these mutations in other populations has shown that the D154N and D206E mutations in exon 4 of the LDLR gene are likely to be of English origin, while the V408M mutation in exon 9 of the gene originated in the Netherlands.^{8,9}

In this study we investigated the possibility that specific LDLR gene mutations were introduced from the United Kingdom to New Zealand, thereby facilitating diagnosis of FH

at the genomic level. We screened polymerase chain reaction (PCR) amplified¹⁰ genomic DNA from 14 unrelated subjects with hypercholesterolaemia originating from the United Kingdom for mutations in exon 4 of the LDLR gene. This gene region appears to be particularly mutation prone and was found to be mutated in about 8% of FH patients in the United Kingdom.^{8,11}

Materials and methods

PATIENTS

The patient sample consisted of 14 apparently unrelated hypercholesterolaemic subjects (nine males, five females) attending the Lipid Disorders Clinic at the Christchurch Hospital in New Zealand (table). These study participants, originating from the United Kingdom, were diagnosed as FH heterozygotes based on the presence of tendon xanthomata and a positive family history of hypercholesterolaemia consistent with autosomal dominant inheritance. All patients had received previous extensive dietary counselling, and were stabilised on diets containing <30% of total energy as fat. All patients were previously using hypolipidaemic drug therapy, which was withdrawn six weeks before baseline blood tests. All patients had normal renal, hepatic, and thyroid function. Blood samples for measurement of lipids were obtained after a 12 hour fast following six weeks on diet alone.

PLASMA LIPID AND LIPOPROTEIN

DETERMINATIONS

Levels of total plasma cholesterol (TC), total triglycerides (TG), and HDL cholesterol (HDL) were determined in the Hunter-Nye Lipid Laboratory in Dunedin (NZ). TC and TG were measured in plasma and plasma fractions using enzymatic kits (Boehringer Mannheim) and controls from the Australian Lipid Standardisation programme. HDL was measured in the supernatant obtained after precipitation of lipoproteins containing apolipoprotein B from plasma. Plasma LDL cholesterol (LDL) was calculated by the Friedewald equation ($LDL = TC - (HDL + TG/2.18)$).

LOW DENSITY LIPOPROTEIN RECEPTOR FUNCTION

LDLR function was assessed using the method of Cuthbert *et al.*¹² Peripheral blood lym-

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Clinical and demographic characteristics of 14 study participants

Patient	Sex	Age (y)	TC	TG	HDLC	LDLC	XMTA	CHD
(mmol/l)								
FH1	F	69	9.8	1.0	1.8	7.4	+	+
FH2	F	43	8.5	0.9	1.2	6.9	+	-
FH3	M	31	8.9	1.4	1.3	6.9	+	-
FH4	M	55	7.1	1.0	1.0	5.6	+	-
FH5	M	67	7.4	2.0	0.9	5.5	+	+
FH6*	F	62	10.3	1.4	1.0	8.6	+	-
FH7	F	46	7.4	0.8	1.2	5.9	+	-
FH8*	M	44	12.9	1.7	1.2	10.9	+	+
FH9	M	70	10.1	1.2	1.4	8.2	+	-
FH10	M	43	9.8	1.1	1.2	8.1	+	-
FH11	F	22	8.7	1.6	1.8	6.2	+	-
FH12	M	41	9.9	1.8	1.2	7.9	+	-
FH13	M	35	7.3	1.1	1.4	5.4	+	-
FH14	M	38	11.6	1.8	1.1	9.6	+	+

* Molecularly characterised FH heterozygotes.

Diet treated lipid levels are given for total cholesterol (TC), triglycerides (TG), high density lipoprotein cholesterol (HDLC), and low density lipoprotein cholesterol (LDLC).

XMTA, tendon xanthomas; CHD, coronary heart disease.

phocytes were isolated from 10 ml of EDTA anticoagulated blood. In each well 5×10^3 cells were cultured in RPMI 1640 medium supplemented with 1% lipoprotein poor plasma and 0.5 $\mu\text{g/ml}$ of phytohaemagglutinin. Mevinolin (0.5 $\mu\text{mol/l}$) was included in all cultures except for control wells which contained DMSO. Human LDL isolated from normocholesterolaemic subjects was added at concentrations between 0 and 5 $\mu\text{g/l}$ to triplicate wells. Each control culture was treated identically and with similar concentrations of LDL. After 96 hours incubation, 1 μCi [^3H]-thymidine was added and a further 18 hour incubation was carried out. Cells were then harvested and [^3H]-thymidine incorporation into DNA was measured by scintillation counting for one minute.

The percentage of mevinolin inhibition was calculated and data were plotted using the sigma Plot computer program. The assay design allowed the measurement of receptor function from lymphocytes isolated from a maximum of three different hyperlipidaemic subjects together with an internal control provided by a healthy normocholesterolaemic subject. Patients with normal LDLR function (four of the initial 18 study participants) were excluded from this study and were screened for mutations in the apolipoprotein (apo) B-100 gene^{13,14} causing familial defective apolipoprotein B-100 (FDB).

ANALYSIS OF GENOMIC DNA

Genomic DNA was extracted from peripheral blood samples and exon 4 of the LDLR gene amplified by the PCR method¹⁰ using two pairs of oligonucleotide primers^{5,15,16}:

5' half: 5'-CATCCATCCCTGCAGCCCC-3' (H)

5'-GGGGTCGTTGTGCGCAGGCC-3' (4.3)

3' half: 5'-CGAGGCCTCCTGCCCGGTGCTCACC-3' (4.2)

5'-GGGACCCAGGACAGGTGATAGGAC-3' (U).

PCR conditions were according to Kotze *et al.*⁵ except that the 5' half of exon 4 was amplified at an annealing temperature of 63°C.

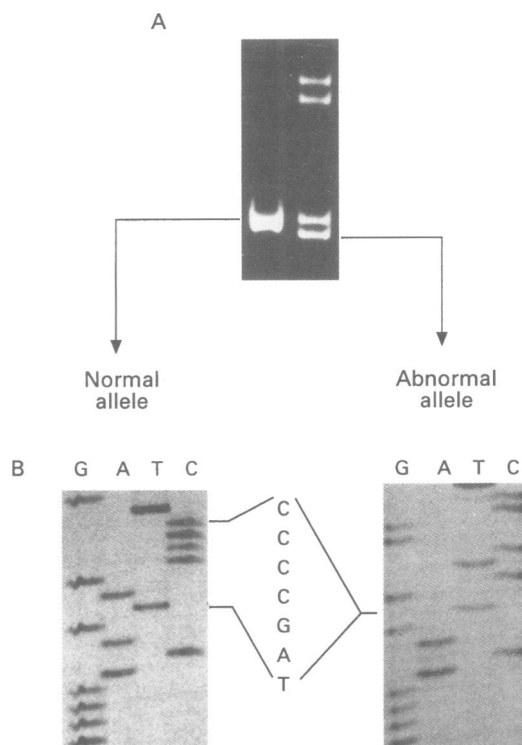
Aliquots of amplified 3' exon 4 DNA of the LDLR gene were digested with *DdeI*, *MboII*, or *MspI*, in order to screen for the D206E,

D154N, or D200G mutations respectively.^{5,11} Samples were electrophoresed for two hours at 100 V on 3% Metaphor gels (FMC Bio-products), stained with ethidium bromide, and photographed under ultraviolet light.

Directly after PCR amplification, 5 μl aliquots of the 5' half and 3' half exon 4 LDLR gene products from subjects normal for the three above mentioned mutations were mixed with an equal volume of gel loading buffer (95% formamide, 20 mmol/l EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol) and electrophoresed on a 30 cm vertical and 1 mm thick low cross linking (1%C) 10% polyacrylamide gel supplemented with 15% urea.¹⁷ Electrophoresis was performed in 0.6 TBE at room temperature (<30°C) at 800 V for five to six hours. The gel was stained in a solution of 0.6 \times TBE containing 1 $\mu\text{g ml}^{-1}$ ethidium bromide.

After detection of altered heteroduplex bands characteristic of a small deletion in the 3' half of exon 4 in one subject, the remaining PCR product was electrophoresed overnight on a standard 5% polyacrylamide gel at 150 V to obtain adequate separation of the normal and mutant alleles. The faster migrating mutant allele (homoduplex) was excised from the gel and eluted overnight in TE (10 mmol/l Tris-Cl, 1 mmol/l EDTA (pH 8.0)). Before reamplification of an aliquot of the eluate with the set of 3' half primers (see above), primer 4.2 was phosphorylated at its 5' end. Single stranded templates were prepared by using a PCR preparation kit according to the manufacturer's (Pharmacia Biotechnology, Uppsala, Sweden) instructions and sequenced with T7 DNA polymerase (T7 sequencing kit, Pharmacia Biotechnology).

Haplotype analyses were performed using four intragenic LDLR gene polymorphisms: the *SmaI* restriction fragment length polymorphism (RFLP) in intron 7,¹⁸ the *StuI* RFLP in exon 8,¹⁹ the *AvaII* RFLP in exon 13,²⁰ and the *NcoI* RFLP in exon 18.²¹ Genotypes at the polymorphic sites were determined by restriction enzyme digestion of PCR amplified DNA and gel electrophoresis. FH associated haplotypes were deduced by homozygosity for the polymorphisms analysed.



Detection and characterisation of the mutation in exon 4 of the LDLR gene. (A) Heteroduplex analysis in a polyacrylamide gel. (B) Allele specific sequencing. Left, sequence of a normal control; right, sequence of the abnormal allele of patient FH8.

Results

The clinical and demographic characteristics of the study population are summarised in the table. All 14 patients showed reduced receptor function consistent with heterozygous FH. Analysis of band patterns of PCR amplified exon 4 DNA of the patients after digestion with *DdeI*, *MboII*, or *MspI* showed that FH6 was heterozygous for the D206E mutation. Genotyping at four polymorphic sites showed that this patient was homozygous for the *SmaI* (+ +), *StuI* (+ +) and, *AvaII* (– –) RFLPs, and heterozygous for the *NcoI* (+ –) RFLP. The chromosomal background of the base change at nucleotide 681 was thus compatible with the originally described mutant allele.

Heteroduplex analysis of PCR products amplified from the 5' half (215 bp) and 3' half (330 bp) of exon 4 did not show altered mobility bands characteristic of previously described mutations. In FH8, however, analysis of the 330 bp fragment showed two slower migrating heteroduplex bands and one faster migrating homoduplex band (figure). Direct DNA sequence analysis of the faster migrating mutant allele of this patient showed a 7 bp deletion (TAGCCCC or AGCCCCT) between nucleotides 581 (or 582) and 589 (or 590) (figure). This deletion causes a frameshift mutation, whereby a truncated protein of the first 173 residues of the LDLR with an additional eight abnormal residues (Ala-Arg-Pro-Ser-Ser-Ser-Thr-Ala) and a premature stop codon (codon 182) would be created. FH8 was homozygous for all four RFLPs analysed, indicating that the 7 bp deletion is associated

with haplotype *SmaI* – / *StuI* + / *AvaII* + / *NcoI* + of the LDLR gene.

Discussion

We screened 14 New Zealand patients presenting with primary hypercholesterolaemia and clinical signs of cholesterol deposition for mutations in exon 4 of the LDLR gene. Mutations in this region of the gene were found to underly FH in about 8% of patients in the United Kingdom,^{8,11} from where the study population originated. Furthermore, more than 20% of the point mutations and small rearrangements reported to date reside in exon 4,²² including the first molecularly characterised de novo mutation.²³

In our study population one patient was heterozygous for the previously described D206E mutation, and her genotype was consistent with the mutation being carried on the same haplotype as was reported originally in a South African FH homozygote.^{15,24} This mutation, occurring in the majority of affected Afrikaners (~60%),⁵ gives rise to a relatively mild form of FH.²⁵ Subsequent detection of this mutation in FH patients of English ancestry in the United Kingdom,⁸ the Netherlands,⁹ North America,²² and now also in New Zealand, indicates that the mutation originated in the United Kingdom. This view is further supported by the haplotype analyses that were conducted in the respective studies.

The new mutation identified in exon 4 involves a 7 bp deletion after nucleotide 581 (or 582) in the LDLR gene. This mutation has not been observed in 500 normal chromosomes screened by heteroduplex analysis (data not shown), and is consistent with the FH phenotype and reduced receptor function observed in FH8. If the RNA is spliced normally, the deletion can be expected to lead to a frameshift and stop codon, and the resulting truncated protein is likely to be rapidly degraded in the cell. Further evidence for the dysfunctional nature of the deletion mutation is the fact that it occurred in the highly conserved apolipoprotein (apo) E/apo B binding repeat 5 of the LDLR.²⁶ Repeat 5 is unique among the seven ligand binding repeats in that its structural integrity is required for binding of both the receptor's ligands. Binding of apo B-100 of LDL requires the integrity of repeats 2 to 7, whereas binding of apo E of β -VLDL is determined predominantly by repeat 5.²⁷

In summary, two of the 14 FH patients analysed in this study were heterozygous for mutations in exon 4 of the LDLR gene, and haplotype data indicated that mutation D206E was introduced from the United Kingdom to New Zealand. Of the initial study population of 18 lipid clinic patients, three clinically affected cases exhibiting normal LDLR activity were heterozygous for the apo B₃₅₀₀ mutation causing FDB (H Lu *et al.*, submitted). These resulted in a total of 5/18 (28%) patients having the molecular basis of their hypercholesterolaemia determined. Owing to the small numbers analysed, we cannot deduce whether the mutations in the study population are representative of

those that will be found in other FH patients in New Zealand. Future screening for the new 7 bp deletion identified in this study, as well as the point mutations detected in the LDLR and apo B genes, will allow frequency determination of the respective mutations. Furthermore, DNA screening of additional family members for known mutations will be of particular importance in children and young adults without tendon xanthomas, where prevention of symptomatic FH can be achieved by lowering of CHD risk factors early in life. Such screening can be performed cost effectively by heteroduplex analysis in low cross linked polyacrylamide gels.^{17,28}

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- 1 Goldstein JL, Brown MS. Familial hypercholesterolemia. In: Scriver CR, Beaudet AL, Sly WS, Valle D, eds. *The metabolic basis of inherited disease*. 6th ed. New York: McGraw-Hill, 1989:1215-50.
- 2 Hobbs HH, Brown MS, Russell DW, Davignon J, Goldstein JL. Deletion in the gene for the low-density lipoprotein receptor in a majority of French Canadians with familial hypercholesterolemia. *N Engl J Med* 1987;317:734-7.
- 3 Lehrman MA, Schneider WJ, Brown MS, et al. The Lebanese allele at the LDL receptor gene locus: nonsense mutation produces truncated receptor that is retained in the endoplasmic reticulum. *J Biol Chem* 1987;262:401-10.
- 4 Aalto-Setälä K, Helve E, Kovanen PT, Kontula K. Finnish type of low density lipoprotein receptor gene mutation (FH-Helsinki) deletes exons encoding the carboxyl-terminal part of the receptor and creates an internalization-defective phenotype. *J Clin Invest* 1989;84:499-505.
- 5 Kotze MJ, Langenhoven E, Warnich L, du Plessis L, Retief AE. The molecular basis and diagnosis of familial hypercholesterolemia in South African Afrikaners. *Ann Hum Genet* 1991;55:115-21.
- 6 Meiner V, Landsberger D, Berkman N, et al. A common Lithuanian mutation causing familial hypercholesterolemia in Ashkenazi Jews. *Am J Hum Genet* 1991;49:443-9.
- 7 Kotze MJ, Langenhoven E, Theart L, Marx MP, Oosthuizen CJJ. Report on a molecular diagnostic service for familial hypercholesterolemia in Afrikaners. *Genet Couns* 1994;5:15-21.
- 8 Gudnason V, King-Underwood L, Seed M, Sun X-M, Soutar AK, Humphries SE. Identification of recurrent and novel mutations in exon 4 of the LDL receptor gene in patients with familial hypercholesterolemia in the United Kingdom. *Arterioscler Thromb* 1993;13:56-63.
- 9 Defesche JC, van Diermen DE, Lansberg PJ, et al. South African founder mutations in the low-density lipoprotein receptor gene causing familial hypercholesterolemia in the Dutch population. *Hum Genet* 1993;92:567-70.
- 10 Saiki RK, Gelfand DH, Stoffel S, et al. Primer directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* 1988;239:487-91.
- 11 Gudnason V, Mak YT, Bettridge J, McCarthy SN, Humphries SE. Use of the single-strand conformational polymorphism method to detect recurrent and novel mutations in the low-density lipoprotein receptor gene in patients with familial hypercholesterolemia: detection of a novel mutation Asp₂₀₀-Gly. *Clin Invest* 1993;71:331-7.
- 12 Cuthbert JA, East CA, Bilheimer DW, Lipsky PE. Detection of familial hypercholesterolemia by assaying functional low-density-lipoprotein receptors on lymphocytes. *N Engl J Med* 1986;314:879-83.
- 13 Soria LF, Ludwig EH, Clarke HRG, Vega GL, Grundy SM, McCarthy BJA. Association between a specific apolipoprotein B mutation and familial defective apolipoprotein B-100. *Proc Natl Acad Sci USA* 1989;86:587-91.
- 14 Pullinger CR, Hennessy LK, Love JA, et al. Familial ligand-defective apolipoprotein B: identification of a new mutation that decreases LDL receptor binding affinity. *Circulation* 1993;88:1-322.
- 15 Kotze MJ, Warnich L, Langenhoven E, Du Plessis L, Retief AE. An exon 4 mutation identified in the majority of South African familial hypercholesterolaemics. *J Med Genet* 1990;27:298-302.
- 16 Yamamoto T, Davis CG, Brown MS, et al. The human LDL receptor: a cysteine-rich protein with multiple Alu sequences in its mRNA. *Cell* 1984;39:27-38.
- 17 Peeters AV, Kotze MJ. Improved heteroduplex detection of single-base substitutions in PCR-amplified DNA. *PCR Methods Applic* 1994;4:188-90.
- 18 Jensen LG, Jensen HK, Kjeldsen M, et al. A new, highly informative SmaI polymorphism in intron 7 of the low density lipoprotein receptor (LDLR) gene. *Clin Genet* 1994;45:52-3.
- 19 Kotze MJ, Retief AE, Brink PA, Weich HFH. A DNA polymorphism in the human low-density lipoprotein receptor gene. *S Afr Med J* 1986;70:77-9.
- 20 Hobbs HH, Esser V, Russell DW, AvaII polymorphism in the human LDL receptor gene. *Nucleic Acids Res* 1987;15:379.
- 21 Kotze MJ, Langenhoven E, Dietzsch E, Retief AE. RFLP associated with the low-density lipoprotein receptor gene (LDLR). *Nucleic Acids Res* 1987;15:376.
- 22 Hobbs HH, Brown MS, Goldstein JL. Molecular genetics of the LDL receptor gene in familial hypercholesterolemia. *Hum Mutat* 1992;1:445-66.
- 23 Kotze MJ, Theart L, Peeters A, Langenhoven E. A de novo duplication in the low density lipoprotein receptor gene. *Hum Mutat* 1994;4.
- 24 Kotze MJ, Langenhoven E, Retief AE, et al. Haplotypes identified by 10 DNA restriction fragment length polymorphisms at the human low density lipoprotein receptor locus. *J Med Genet* 1989;26:255-9.
- 25 Kotze MJ, de Villiers WJS, Steyn K, et al. Phenotypic variation among familial hypercholesterolemic heterozygous for either one of two Afrikaner founder LDL receptor mutations. *Arterioscler Thromb* 1993;13:1460-8.
- 26 Mehta KD, Chen WJ, Goldstein JL, Brown MS. The low density lipoprotein receptor in *Xenopus laevis*. *J Biol Chem* 1991;266:10406-14.
- 27 Russell DW, Brown MS, Goldstein JL. Different combinations of cysteine-rich repeats mediate binding of low density lipoprotein receptor to two different proteins. *J Biol Chem* 1989;264:21682-8.
- 28 Kotze MJ, Langenhoven E, Peeters AV, Theart L, Oosthuizen CJJ. Detection of two point mutations causing familial defective apolipoprotein B-100 by heteroduplex analysis. *Mol Cell Probes* 1994;8:513-18.