Identification of a common low density lipoprotein receptor mutation (C163Y) in the west of Scotland

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Abstract

Familial hypercholesterolaemia (FH) is an autosomal codominant disorder characterised by high levels of LDL cholesterol and a high incidence of coronary artery disease. Our aims were to track the low density lipoprotein receptor (LDLR) gene in individual families with phenotypic FH and to identify and characterise any mutations of the LDLR gene that may be common in the west of Scotland FH population using single strand conformational polymorphism analysis (SSCP). Patient samples consisted of 80 heterozygous probands with FH, 200 subjects who were related to the probands, and a further 50 normal, unrelated control subjects. Tracking of the LDLR gene was accomplished by amplification of a 19 allele tetranucleotide microsatellite that is tightly linked to the LDLR gene locus. Primers specific for exon 4 of the LDLR gene were used to amplify genomic DNA and used for SSCP analysis. Any PCR products with different migration patterns as assessed by SSCP were then sequenced directly. In addition to identifying probands with a common mutation, family members were screened using a forced restriction site assay and analysed using microplate array diagonal gel electrophoresis (MADGE). Microsatellite D19S394 analysis was informative in 20 of 23 families studied. In these families there was no inconsistency with segregation of the FH phenotype with the LDLR locus. Of the FH probands, 15/80 had a mutant allele as assessed by SSCP using three pairs of primers covering the whole of exon 4 of the LDLR gene. Direct DNA sequencing showed that 7/15 of the probands had a C163Y mutation. Using a PCR induced restriction site assay for the enzyme *RsaI* and MADGE, it was determined that the C163Y mutation cosegregated with the FH phenotype in family members of the FH probands. This mutant allele was not present in any of the control subjects. Microsatellite analysis has proven useful in tracking the LDLR gene and could be used in conjunction with LDL cholesterol levels to diagnose FH, especially in children and young adults where phenotypic diagnosis can be difficult.

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Familial hypercholesterolaemia (FH) is an autosomal codominant disorder, characterised by a two to three fold rise in low density lipoprotein (LDL) cholesterol, tendon xanthoma, and an increased risk of myocardial infarction with a variable age of onset.¹ In its heterozygous form it affects 1/500 people, suggesting that there are more than 110 000 FH heterozygotes in the UK alone, of which probably only 5% have been diagnosed at the clinical level and only 0.1% at the molecular level (see website: <http://www.ucl.ac.uk/fh>). LDL are catabolised primarily via the LDL receptor pathway mediated by the interaction between the LDL receptor (LDLR) and apolipoprotein B (apoB). Mutations within the LDLR gene disrupt this interaction and cause the phenotype of FH. Over 300 different mutations of the LDLR gene, which spans 45 kb and comprises 18 introns and 17 exons, have been characterised at the molecular level.² Such mutations include both small and large deletions, insertions, and single base pair substitutions.³⁻⁶ The large interpersonal variability in phenotypic expression of FH could be explained by the underlying mutation that is present in the affected subjects,⁷⁻⁹ though other genetic influences such as apoE polymorphism or the presence of a missense mutation in the LDLR binding region of apoB may also play an important role.¹⁰⁻¹² Previous studies have shown that within geographically or culturally isolated populations or where a large population is related by descent, the frequency of certain mutations is increased.¹³⁻¹⁴ This is illustrated by the French Canadian population where a 10 kb deletion at the 5' end of the LDLR gene accounts for 60% of the FH population and, even more strikingly, 100% of the Lebanese FH cases studied had a simple base pair substitution which results in the formation of a stop codon and ultimately a truncated form of the LDL receptor with less than 2% LDLR activity.¹⁵⁻¹⁶

The large size of the LDLR gene in conjunction with the numerous mutations so far characterised makes the detection of mutations in the LDLR gene difficult. A large number of mutations, such as large insertions and deletions, have been detected using restriction fragment length polymorphism (RFLP) analysis and Southern blotting.¹⁷ A newer method,

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Table 1 Clinical data for FH probands for C163Y mutation. Molecular data for relatives of probands also included assessed by both direct sequencing and forced restriction site assay using the MADGE system

Proband	Age (y)	Gender (M/F)	Total chol (mmol/l)	Fasting trig (mmol/l)	LDL-C (mmol/l)	HDL-C (mmol/l)	C163Y	D19S394 allele sizes (NT)	No of relatives reviewed	No of relatives positive for FH by TC	No of relatives C163Y positive by direct sequencing	No of relatives C163Y positive by MADGE
07	32	F	8.6	0.75	7.1	1.15	+	231/251	4	2	2	2
09	68	M	11.6	1.6	8.84	1.26	+	227/259	6	2	2	2
26	63	F	9.4	1.25	7.8	1.3	+	227/227	0	—	—	—
32	44	M	9.9	1.5	8.1	1.45	+	215/227	6	3	3	3
37	46	M	12.9	1.8	10.9	0.7	+	227/235	0	—	—	—
59	61	M	8.4	1.2	6.5	1.4	+	227/255	10	4	4	4
85	38	M	11.5	1.6	9.97	0.63	+	235/267	0	—	—	—

single strand conformational polymorphism analysis (SSCP), is based on the ability of denatured DNA to assume a three dimensional conformation which is dependent on its primary sequence.¹⁸ If a mutation exists between the wild type and mutant DNA, this will result in differential migration on a polyacrylamide gel. The heterogeneity of the FH population in the UK would suggest that

any one mutation occurring at a high frequency would be unlikely. In a sample of 200 FH patients from London, 5% have been shown to have a gross rearrangement and a further 18% a small deletion or single base substitution on exons 3, 4, or 14 of the LDLR gene.^{19,20} However, Day *et al*²¹ have recently shown that a common mutation (R329X) accounts for 11.5% in Southampton and south west Hampshire.²¹ There is evidence that hypercholesterolaemia associated with early morbidity and mortality from coronary heart disease is common in the west of Scotland, but there have been no previous systematic studies on phenotype/genotype relationship in this population.

The objectives of this study were three fold: firstly, to track the LDLR gene in families with FH using the highly polymorphic marker D19S394 on chromosome 19p, and to test whether this mutation can be used in conjunction with LDL cholesterol levels to diagnose FH, especially in children and young adults where phenotypic diagnosis can be difficult; secondly, to identify and characterise any LDLR gene mutations that may be common in the west of Scotland using SSCP analysis followed by direct sequencing; and, thirdly, to develop a PCR based restriction site assay compatible with microplate array diagonal gel electrophoresis for any common LDLR mutations found.

Material and methods

PATIENTS

Patient samples included 80 probands with FH (all apparently unrelated), 200 subjects related to the probands, and 50 normal controls. Clinical criteria for FH were a documented fasting total cholesterol >9 mmol/l and a LDL cholesterol level greater than 7 mmol/l and one of the following indices: a strong family history of coronary heart disease, the presence of either tendon xanthoma or xanthelasma, or a personal history of heart disease. FH probands were recruited from the Glasgow Royal Infirmary and Western Infirmary lipid clinics. Secondary hyperlipidaemia was excluded by selecting subjects with normal γ -glutamyltransferase (γ -GT), normal thyroid function tests, and no history of diabetes mellitus. Patients with familial defective apoB were excluded by testing for the apoB-3500 as previously described. Ethical approval was obtained from the Ethics Committees of both hospitals and all subjects gave their informed consent.

Figure 1 (A) SSCP gel to detect mutations in the mid portion of exon 4 of the LDLR gene. Lanes 1-12 SSCP migration pattern of 12 unrelated patients with a clinical diagnosis of definite or probable FH. Lanes 13-24 SSCP migration pattern of 12 control patients. Gel run at 300 volts for 16 hours at 25°C. Lanes 2, 5, and 10 show FH probands with abnormal migration patterns. (B) Sequence analysis of amplified mid portion of exon 4 of the LDLR gene in a patient who is heterozygous for the C163Y mutation. (C) Sequence analysis of amplified 3' end of exon 4 of the LDLR gene in a patient who is heterozygous for the C210X mutation. Sequence analysis of other mutations detected by SSCP not shown.

Table 2 Direct DNA sequencing in 15 probands with different migration patterns as assessed by SSCP

No	Mean age	Gender M/F	Exon	Nucleotide change	Codon change	Total chol (mmol/l)	Trigs (mmol/l)	LDL-C (mmol/l)	HDL-chol (mmol/l)
7	50.3 (5.2)	5/2	4mid	TGT→TAT	C163Y	10.4 (0.6)	1.4 (0.1)	8.5 (0.6)	1.1 (0.1)
3	31 (5.7)	2/1	45'	TGC→TGA	C210X	8.5 (0.1)	1.2 (0.4)	6.7 (0.1)	1.2 (0.1)
1	34	1/0	45'	GAG→TAG	E207X	9.3	0.8	7.6	1.0
1	52	1/0	4mid	GAC→AAC	D147N	8.0	1.8	5.8	0.9
1	41	1/0	45'	GGC→GAC	G198D	8.7	1.1	6.8	0.7
1	48	0/1	4mid	⁵⁵⁸ CCC→ ⁵⁵⁸ CC	513dC	10.0	1.9	7.5	0.8
1	52	0/1	45'	⁵¹¹ CCC→ ⁵¹¹ CC	660dC	12.1	1.5	8.6	1.2

Values given in brackets are expressed as SEM.

PURIFICATION OF GENOMIC DNA

Genomic DNA from probands, relatives, and normal subjects was purified from peripheral leucocytes as described by Hobbs *et al.*²²

SSCP ANALYSIS AND SEQUENCING

Exon 4 of the LDLR gene was amplified by polymerase chain reaction (PCR) using three pairs of oligonucleotide primers. Upstream primer 5'-GTTGGGAGACTTCACACGGT GATGG-3' and downstream primer 5'-ACCGGGCAGGAGGCCGCTCT-3' giving a 171 bp fragment, corresponding to the 5' end of exon 4. Upstream primer 5'-AGACGCGGCCTCCTGCCCCGGT-3' and downstream primer 5'-GAGCAGGGGCTAC TGTCC-3' giving a 180 bp fragment, corresponding to the mid portion of exon 4. Upstream primer 5'-GGACAGTAGCCCCCT GCTCG-3' and downstream primer 5'-GGAGCCCAGGGACAGGTGATAG-3' giving a 175 bp fragment, corresponding to the 3' end of exon 4. Primers were from Cruachem

Ltd, UK. Primers were labelled using T4 polynucleotide kinase (New England Biolabs, UK) and γ^{32} P ATP and used for subsequent amplifications of genomic DNA. The amplifications were performed on a thermal cycler (DNA Engine, MJ Research, UK) using *Thermus aquaticus* DNA polymerase (Promega, UK) in a buffer recommended by the manufacturer and a total volume of 20 μ l. The conditions were 95°C for five minutes once and subsequently 95°C for one minute and 61°C (5' end primers), 57°C (mid portion primers), or 68°C (3' end primers) for one minute and 72°C for one minute for 30 cycles.

A quantity of 5 μ l PCR products and 12 μ l stop buffer (95% formamide, 20 nmol/l EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol) were mixed and denatured at 95°C for three minutes and snap cooled on ice. Samples (4 μ l) were loaded onto a 6% non-denaturing polyacrylamide gel (ratio of acrylamide to bisacrylamide 49:1, Severn Bio- tech, UK), with or without 10% glycerol and

Figure 2 (A-C) Examples of three kindreds with the C163Y mutation detected by SSCP analysis. Direct sequencing of the samples was carried out first and then confirmed using the forced restriction site assay with the enzyme *RsaI* and using the MADGE system. The figures show that in these families the 227nt allele cosegregates with the C163Y mutation. y=years of age, eg 68 y, TC=total cholesterol (mmol/l), D19S394 genotypes are given as allele sizes in nucleotides, eg 227 259, +/- C163Y indicates the presence or absence of the mutation confirmed by sequencing then forced restriction sites assay for *RsaI*.

Figure 3 (A) PCR induced *RsaI* restriction site assay for the C163Y mutation analysed by microplate array diagonal gel electrophoresis (MADGE). (B) Schematic of MADGE; a known proband for the mutation C163Y (well B1) was included as well as an unrelated control subject (well C2). Two PCR patterns were observed dependent on the absence or presence of the C163Y mutation (C). In the absence of the mutation, complete digestion of the PCR products was achieved resulting in two bands being present (40+24 bp) when visualised by the MADGE system. In the presence of the mutation, partial digestion was achieved resulting in three bands being present (64+40+24 bp).

run at either room temperature for 16 hours at 20 mA or at 4°C for four hours at 45 mA. After electrophoresis, the gel was dried and exposed to hyperfilm B max (Amersham, UK) for 24 to 48 hours.

Sequencing was used to confirm any mutations observed by SSCP analysis. PCR products were purified using a gel nebuliser and amicon columns of the appropriate bands from a 1% agarose gel. The purified PCR products were then sequenced using an Amplicycle sequencing kit (Perkin Elmer, UK).

ANALYSIS OF POLYMORPHIC MICROSATELLITE MARKER D19S394

PCR oligonucleotide sequences for the tetranucleotide repeat marker D19S394 were as follows 5'-FAM-AGACTACAGTGACTGTGG-3' for the sense primer and 5'-GTGTTCCCTAACTCCAGGC-3' for the antisense primer. Primers were from PE Applied Biosystems, UK). Optimal PCR conditions have been described previously by Day *et al.*²¹

RESTRICTION SITE ASSAYS FOR C163Y

No restriction enzyme cutting site was found in the sequence of the wild type or mutant for the

substitution of G to A at position 551 in which the wild type sequence was ⁵⁴⁸GCTGTAG⁵⁵⁴ mutated to ⁵⁴⁸GCTATAG⁵⁵⁴. It was therefore necessary to carry out PCR by using one mismatched primer (sense primer 5'-CGAC TGCGAAGATGGCTCG-3', antisense mismatched primer 5'-CCCTTGGGAACACGT AAAGACCCGTA-3') to introduce a C at position 554, thereby introducing a new restriction site for the enzyme *RsaI* (Boehringer Mannheim, UK) and yielding a 64 bp product. PCR products (8 µl) were added to a digestion mix (2 µl) containing the restriction enzyme *RsaI* and buffer L, leading to the complete digestion of wild type PCR product resulting in a 40 and 24 bp fragment, whereas the presence of the C163Y mutation would result in only partial digestion and result in the production of 64, 40, and 24 bp fragments. Digested PCR products (5 µl) were added to loading buffer (2 µl) and analysed using polyacrylamide microplate array diagonal gel electrophoresis (MADGE).

STATISTICAL METHODS

Statistical analysis was carried out using Fisher's exact test.

Results

Genomic DNA from 80 patients with familial hypercholesterolaemia from the west of Scotland, all apparently unrelated, were subjected to analyses by SSCP of exon 4 of the LDL receptor. Any differential patterns detected by SSCP were then sequenced directly. Fifteen FH probands had different migration patterns as assessed by SSCP (fig 1). Direct sequencing showed that seven of the 15 probands with the abnormal SSCP pattern had a substitution of G→A that changes codon 163 from cysteine to tryptophan (C163Y). Direct sequencing also showed that in three other patients the abnormal SSCP pattern was the result of the substitution of C→A, changing codon 210 from cysteine to a stop codon (C210X). Clinical details of the probands with mutant alleles C163Y and C210X are shown in tables 1 and 2, respectively. In addition to identifying the C163Y and C210X mutation, several other mutations were also identified in eight other FH probands; these included E207X, D147N, G198D, 513delC, and 660delC. With exception of the 513delC and 660delC mutations, the other mutations have been reported by other groups.

From three of the FH probands with the C163Y mutation, additional family members were available. These family members were subjected to direct sequencing to establish the presence or absence of the C163Y mutation of the LDLR gene. From the first family studied, one of the sisters of the proband showed the presence of the C163Y mutation and had a raised cholesterol, whereas the remaining sibs did not have the mutation and had normal cholesterol levels (fig 2A). In the second family, three relatives of the proband had the C163Y mutation and a raised cholesterol (fig 2B); other family members tested for the mutation were negative for the C163Y mutation. In the

Figure 4 Distribution of the allele sizes for the microsatellite marker D19S394 for the control and FH groups from the west of Scotland.

third family, 10 family members were available, of whom four members had the C163Y mutation and raised cholesterol (fig 2C). As well as direct sequencing, a direct assay using PCR to induce a *RsaI* restriction site was also tested, with the future aim of using MADGE as a screening method for the C163Y mutation in a large population of subjects from the west of Scotland. This direct assay method was fully validated against direct sequencing (fig 3).

In conjunction with using SSCP, the tetranucleotide microsatellite marker D19S394 was also used to track the LDL receptor within individual families. The heterozygosity of the marker was greater than 0.9 in our control population from the west of Scotland. In this study the inheritance of the LDL receptor could be established in 20 out of 23 families where there were large kindreds. Of the seven probands with the C163Y mutation, the D19S394 allele size 227nt cosegregated with the FH phenotype in five of the probands, although it occurs in only 4% of control chromosomes (fig 4); comparison of the allele frequency of 227nt in the probands with C163Y to the frequency in the population by Fisher's exact test indicates that these are indeed significantly different ($p=0.0001$).

Discussion

The primary criterion for diagnosis of FH is that plasma lipid levels are raised, though this can be misleading since there is considerable overlap of plasma lipid levels between FH heterozygotes and those of the general population.²³⁻²⁵ The large number of mutations that have been identified so far means that the use of molecular tools is inefficient and that no mutation analysis technique is believed to be 100% sensitive. With the development of PCR technology such as SSCP, denaturing gradient gel electrophoresis (DGGE), and a new oligo ligation assay (OLA), it has become easier to identify a specific mutation of the LDL receptor at the molecular level in a number of subjects with FH on the assumption that a common founder mutation is present within a certain population.^{6 8 26-28} Our present study has tried to identify whether a common mutation exists in the west of Scotland FH population. We have identified a single base substitution

(G→A) in the mid portion of exon 4 of the LDLR that converts the amino acid cysteine 163 to tryptophan; this mutation occurs in 8.75% of our FH population and has been previously reported to occur in the Northern Irish population (website; <http://www.ucl.ac.uk/fh>). Mutations within the mid portion of exon 4 of the LDLR gene are functionally significant since this region codes for the ligand binding domain of the LDLR. The increased frequency of this mutation would suggest that a direct assay using PCR would prove helpful in diagnosing affected family members, especially in young children where it can be difficult to diagnose FH using clinical and biochemical data alone. SSCP is a relatively simple method which makes it a powerful tool for detecting mutations but can be rather time consuming, and this is complicated by the fact that direct sequencing of any mutant alleles detected by SSCP would be required. The assay for introducing the restriction site for *RsaI* would prove useful in mass screening for the C163Y mutation in the FH population. It has been reported that even though the UK FH population is heterogeneous in its ancestry, regional variation of certain mutations of the LDLR gene occurs.^{3 21} This has been shown in the FH population of the Manchester area where one such mutation (E80K) occurs in 10% of probands studied, with the mutation itself being widely distributed in the UK²⁹ with a more typical prevalence of 1-2%. Recently it has been reported that in the south of England the mutation R329X also occurs in 10% of FH kindreds, though prevalence elsewhere is unknown. Cosegregation analysis in three of our families would suggest that the C163Y mutation described in the current study is responsible for the raised cholesterol observed in these seven FH probands.

It has recently been shown that an allele of the microsatellite marker D19S394 is in complete linkage disequilibrium with the LDLR mutation R329X. The authors have suggested that the use of this microsatellite marker would enable the rapid recognition of a possible common FH mutation within a group of FH patients from either a particular geographical location or a common ancestry.²¹ The marker is approximately 250 kb 5' to the LDL receptor gene suggesting that recombination would be relatively rare between the marker and the LDLR in a single generation. In our study, the marker was used to track the LDL receptor within individual families and alleviated the problems associated with using haplotype analysis and RFLPs. Of the seven patients with the C163Y mutation, five have one or more D19S394 227 bp alleles, compatible with the mutation having occurred relatively recently on a chromosome with this length, with this allelic association being maintained because of the small genetic distance, and thus the low rate of recombination, between the LDLR locus and the D19S394 locus. The remaining two probands have the microsatellite genotype 231/251 and 235/267, and this raises the possibility that the mutation may have occurred independently on at least

two other chromosomes. We consider this to be unlikely since the G→A change that creates the C163Y mutation is not at a CpG mutational hot spot. Microsatellites are known to mutate primarily by the process of replication slippage,³⁰ and this would result in the loss of allelic association. These possibilities could be examined in these patients by determining haplotypes using RFLPs within and flanking the LDL locus, but such data were not available for these subjects.

Among FH patients there is a great deal of variation in terms of the circulating lipid levels and the age of onset of coronary heart disease. To date no association between specific mutations of the LDL receptor gene, the severity of clinical presentation, and the natural history of the disease has been observed. However, it has been shown that the age of onset of CAD aggregates within families.³¹ This may be related to the presence of both environmental influences and genetic factors such as a given LDLR mutation, lipoprotein (a), and apoE genotype. If such genotype/phenotype relationships could be established for specific mutations, then more active/aggressive therapeutic strategies could be applied to both the probands and affected family members.^{32 33}

In conclusion, microsatellite analysis using D19S394 has proven useful in tracking the LDL receptor gene and could be used in conjunction with LDL cholesterol levels as a highly efficient linkage marker in FH kindreds, especially in children and young adults where phenotypic diagnosis can be difficult. SSCP and sequencing analysis of FH probands suggests the existence of a small but significant founder gene effect in the west of Scotland. The development of a direct restriction assay will allow for screening of patients and families at risk and could be applied to any other new mutations that may be detected.

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