

Predominance of a 6 bp deletion in exon 2 of the LDL receptor gene in Africans with familial hypercholesterolaemia

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Abstract

In South Africa, the high prevalence of familial hypercholesterolaemia (FH) among Afrikaners, Jews, and Indians as a result of founder genes is in striking contrast to its reported virtual absence in the black population in general. In this study, the molecular basis of primary hypercholesterolaemia was studied in 16 Africans diagnosed with FH. DNA analysis using three screening methods resulted in the identification of seven different mutations in the coding region of the low density lipoprotein (LDLR) gene in 10 of the patients analysed. These included a 6 bp deletion (GCGATG) accounting for 28% of defective alleles, and six point mutations (D151H, R232W, R385Q, E387K, P678L, and R793Q) detected in single families. The Sotho patient with missense mutation R232W was also heterozygous for a de novo splicing defect 313+1G→A. Several silent mutations/polymorphisms were detected in the LDLR and apolipoprotein B genes, including a base change (g→t) at nucleotide position -175 in the FP2 LDLR regulatory element. This promoter variant was detected at a significantly higher (p<0.05) frequency in FH patients compared to controls and occurred in cis with mutation E387K in one family. Analysis of four intragenic LDLR gene polymorphisms showed that the same chromosomal background was identified at this locus in the four FH patients with the 6 bp deletion. Detection of the 6 bp deletion in Xhosa, Pedi, and Tswana FH patients suggests that it is an ancient mutation predating tribal separation approximately 3000 years ago.

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Autosomal dominant hypercholesterolaemia (ADH) is most commonly caused by mutations in the low density lipoprotein receptor (LDLR) gene causing familial hypercholesterolaemia (FH), or in the apolipoprotein B (ApoB) gene causing familial defective apo B (FDB).^{1,2} These biochemical defects result in the precipitation of excess cholesterol and clinical characteristics include tendon xanthomata and pre-

mature coronary heart disease (CHD). The estimated incidence of both FH and FDB is approximately 1 in 500 in most white populations.

In the Afrikaner population of South Africa, the prevalence of FH has been increased to approximately 1 in 70, as a consequence of a founder effect following the introduction of at least three defective LDLR gene alleles by European settlers.³⁻⁵ This is in striking contrast to the apparently low prevalence of FH in the black population, reported to have migrated from central Africa to the south in three main groups, the Ngunis (Xhosa, Tembu, Swazi, and Zulu) along the east coast, the Sothos (South Sotho, North Sotho/Pedi, West Sotho/Tswana) who settled further west on the Transvaal highveld, and the Vendas living in the northern Transvaal area.^{6,7} We suspect that FH is not frequently recognised in Africans because of altered clinical expression and not because of a lower mutation prevalence compared to most other populations. Previous studies have indicated that the mutational mechanisms giving rise to germline mutations are largely a function of the local DNA sequence environment.⁸⁻¹⁰

Since the situation in South Africa is ideal for studies of underlying lipid related genetic differences among population groups,¹¹ we attempted to identify black hypercholesterolaemics to determine the spectrum of mutations in the promoter and coding region of the LDLR gene and in exon 26 of the ApoB gene. FDB has not previously been studied in the South African black population, but was found to be rare in other South African populations, most likely because of a "negative" founder effect that diluted the frequency of the common ApoB 3500 mutation in the immigrants relative to their parent populations.¹²

Subjects and methods

SUBJECTS

Blood samples were collected from 56 black patients attending lipid clinics in South Africa, after obtaining informed consent and ethical approval from the regional Review Committees. Details on clinical features and ethnicity were provided by the referring clinicians. Sixteen patients with a diagnosis of "classical" or "probable" FH, including two FH homozygotes, were selected for extensive mutation analysis for the coding and promoter region of the LDLR gene and exon 26 of the ApoB gene. Blood samples were also obtained from 38 of their family

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Table 1 Characteristics of African probands analysed for *LDLR* and *apo B* gene mutations

Index	Ancestry	Sex	Age	TC	TG	HDL	LDL	Clinical	<i>LDLR</i> gene sequence changes	<i>Apo B</i> gene sequence changes	Relatives tested
				(mmol/l)‡							
CM	Xhosa	F	52	8.5	2.7	1.5	5.8	CHD	R793Q		0
MX	Xhosa	M	50	10.8	2.0	0.9	9.0	Arc, Xan	-175g→t		0
AN	Swazi	F	58	10.1	0.9	1.3	8.4	Arc, Xan, CHD	D151H		0
AS	Swazi	M	49	8.0	1.0	2.1	5.4	Arc, CHD	P678L		0
AM*	Swazi/Zulu	F	56	8.3	1.5	1.9	5.7	Arc			11
CK†	Zulu	M	26	13.8	0.8	1.3	12.1	Arc, Xan	-175g→t		1
SH	Sotho	M	33	12.7	2.2	1.2	10.5	Arc, Xan	313+1G→A; R232W		4
RK	Sotho	M	58	10.7	2.3	1.0	8.6	CHD	R385Q		0
KN†	Pedi	F	32	14.9	0.8	1.4	13.1	Arc, Xan	6-bp del, 6-bp del		0
EF	Pedi	F	56	13.1	1.1	1.2	11.4	Arc, PVD, CHD	E387K; -175g→t; C347C	T3552T; T3540T	10
LP	Pedi	F	61	9.4	0.8	0.9	8.1	Arc, Xan	6-bp del		3
CN*	Pedi	F	57	7.4	2.6	0.9	5.3	Arc, PVD			6
RM*	Pedi/Tswana	F	54	10.8	0.4	1.3	9.3	Arc, ?Xan			3
LM*	Tswana	F	56	6.1	1.8	1.8	3.5	Arc, CHD	-175g→t	T3552T	0
RL	Tswana	F	30	9.3	0.8	1.7	7.2	Arc, Xan	6-bp del		0
CS	Tswana	F	47	7.9	0.7	1.7	5.9	Arc	6-bp del		0

The majority of mutations summarised in this table were included in a recent mutation update.²²

Reference plasma cholesterol concentrations in the general black population are given in ref 24.

TC, total cholesterol; TG, triglycerides; HDL, high density lipoprotein cholesterol; LDL, low density lipoprotein cholesterol; CHD, coronary heart disease; PVD, peripheral vascular disease; Arc, arcus cornealis; Xan, xanthomata.

*Probable FH.

†Clinical FH homozygotes.

‡Pretreatment concentrations, except for proband LM for whom pretreatment levels were not available.

members (table 1). Classical FH (12 probands) was defined as the occurrence of pretreatment total cholesterol (TC) >7 mmol/l, with the presence of tendon xanthomata or premature CHD or both in the index case or a first degree relative. Probable FH (four probands) was defined by the same pretreatment cholesterol level and primary hypercholesterolaemia or premature CHD or both in the family (table 1). DNA samples of the 40 lipid clinic patients without the FH phenotype, but who had hyperlipidaemia or normal lipid profiles in the presence of vascular disease, were included for analysis of specific regions of the *LDLR* gene. Ninety six people drawn from the same population (19 Pedis, 21 Sothos, 27 Xhosas, and 29 Zulus) were sampled as controls. TC, high density lipoprotein cholesterol (HDL), and triglyceride (TG) determinations and extraction of genomic DNA were performed using standard methods.¹³ Plasma LDL cholesterol (LDL) concentrations were calculated with the Friedewald formula ($LDL = TC - (HDL + TG/2.18)$).¹⁴

MUTATION DETECTION

Heteroduplex single strand conformation polymorphism (HEX-SSCP) analysis was performed in South Africa¹⁵ and denaturing gradient gel electrophoresis (DGGE) in Denmark¹⁶ and Scotland¹⁷ to screen polymerase chain reaction (PCR) amplified genomic DNA for mutations in the *LDLR* and *ApoB* genes. For HEX-SSCP analysis, the exon specific primers described by Jensen *et al*¹⁸ were used, while the promoter region of the *LDLR* gene was amplified using primers 5'-GAGGCAGAGAGGACAATGGC-3' and 5'-CCACGTCATTTACAGCATTTCATG-3'. Base changes in the promoter region were numbered according to Hobbs *et al*,¹⁹ after adding an additional A within the AAAA stretch preceding repeat 1, which is missing from the published sequence.²⁰ PCR products showing aberrant electrophoresis patterns were sequenced on both strands with a PCR

Product Sequencing kit (Amersham) or an automated sequencer ABI 373A or both.

HAPLOTYPE ANALYSIS

Haplotype analysis using four *LDLR* gene polymorphisms was performed according to Theart *et al*.²¹ Microsatellite markers VWA31, F1A1, and TH01 (Profiler kit, Applied Biosystems) were used to test for biological consistency in two families.

STATISTICAL ANALYSIS

Allele frequencies were determined by allele counting. Testing for significance of heterogeneity in mutation frequencies among patient and control groups was based on the chi-square and Fisher's exact tests.

Results

Extensive DNA screening of the *LDLR* gene in 16 black FH patients, using both the DGGE and HEX-SSCP screening methods, showed six missense mutations in individual families and a 6 bp deletion in four probands (table 1).²² The deletion (FH Cape Town-1), previously described in a Xhosa FH homozygote,²³ and missense mutations D151H and R385Q have not (yet) been reported in other populations. Haplotype *SmaI*+/*SnaI*+/*AvaII*- was associated with the deletion in all three FH heterozygotes and a homoallelic FH homozygote. Screening of the coding region in DNA of the four FH patients heterozygous for a base change (g→t) at nucleotide position -175 of the *LDLR* gene promoter resulted in the detection of a recycling deficient mutation E387K¹⁹ in the DNA of subject EF. Interestingly, this Pedi proband was found to be extremely heterogeneous at the DNA level, since a silent C to T base change was furthermore detected at nucleotide position 1104 in exon 8, in addition to two silent mutations in the *ApoB* gene. The G to C change in the third base of codon 3540 (T3540T) and the T to C change in the third base of codon 3552 (T3552T) in the *ApoB* gene have previously been reported in

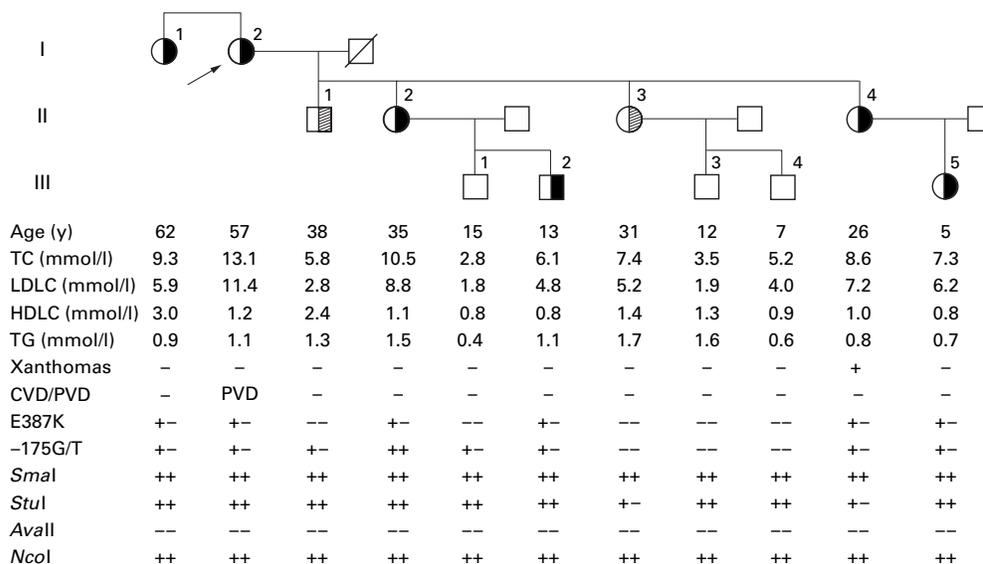


Figure 1 Pedigree of proband EF (arrow) clinically diagnosed with heterozygous FH. Clinical, biochemical, and genetic data are provided for people for whom DNA samples were available. Those with raised plasma cholesterol levels are indicated by dark (mutation positive for E387K) and shaded symbols. The presence (+) or absence (-) of LDLR gene mutations and recognition sites for SmaI, StuI, AvaII, and NcoI are indicated.

Nigerian and African-American subjects, respectively.¹⁷ One of the daughters of proband EF (II.3 in fig 1) carried two copies of the silent *ApoB* mutation at codon 3540. RFLP analysis indicated that haplotype *SmaI*+/*StuI*+/*AvaII*-/*NcoI*+ cosegregated with the -175t allele in the family (fig 1). This chromosomal background was also identified in two of the other probands with the sequence substitution at -175 in the *LDLR* promoter region, while haplotype *SmaI*-/*StuI*+/*AvaII*+/*NcoI*+ was associated with the t allele in the Tswana proband (LM), who also carried the T3552T variant in the *ApoB* gene.

In order to determine whether the two mutations identified in each of probands EF and SH occur in cis or in trans on their respective chromosomes, blood samples were obtained from additional family members for segregation analysis. Pedigree analysis in the family of EF showed that mutation E387K and the -175g→t variant occur on the same chromosome (fig 1). All the family members who inherited the 387K/-175t haplotype (I.1, I.2, II.2, II.4, III.2, and III.5) had abnormally high TC and LDLC levels. II.2, with a clinical diagnosis of heterozygous FH, was homozygous for the t allele at nucleotide position -175. This implies that her deceased father (husband of the index case) also carried the -175g→t promoter variant, but in the absence of mutation E387K. Her normocholesterolaemic son (III.1), as well as her brother, inherited this paternal chromosome, the latter presenting with a moderately raised TC value. The proband's son (II.1) and one of her daughters (II.3) (confirmed by marker studies using highly informative microsatellites) had moderately raised plasma cholesterol concentrations in the absence of either the promoter variant or the exonic mutation, indicating that another unknown factor contributes to the abnormal lipid profile observed in this family. TC concentrations were found to be very low in the

general black population (approximately 3 mmol/l) compared with other South African groups.^{24 25}

DNA screening of the 53 year old father of proband CK, diagnosed with homozygous FH, showed homozygosity for the t allele at nucleotide position -175. His TC and LDLC levels were 6.11 mmol/l and 4.29 mmol/l, respectively, which is comparable to that of a FH heterozygote. Plasma TG and HDLC concentrations were 1.49 mmol/l and 1.14 mmol/l, respectively, and the only clinical feature indicative of hyperlipidaemia in this obligate FH heterozygote was corneal arcus.

HEX-SSCP analysis indicated that the splicing defect identified in exon 3 represents a de novo event in the family of SH, since it was not present in any of his close relatives analysed. Familial relationship was illustrated by transmission of the exon 5 mutation (R232W) from the father (72 years, TC 4.1 mmol/l), and was further substantiated by marker studies using three highly informative microsatellites (data not shown). Mutation R232W was absent in the normocholesterolaemic brother (30 years, TC 3.5 mmol/l) and sister (42 years, TC 3.3 mmol/l) of the proband. Their mother, aged 62 years, presented with a TC level of 2.9 mmol/l. It was therefore not possible to determine whether the splice mutation occurred in cis in the proband on the paternal chromosome bearing mutation R232W, or in trans on the normal maternal chromosome.

Subsequent DNA screening of 96 controls from the general black population comprising 56 Ngunis (27 Xhosas, 29 Zulus) and 40 Sothos (19 Pedis, 21 Sothos) resulted in the identification of six subjects (four Ngunis (one Xhosa, three Zulus) and two Sothos (one Pedi, one Sotho)) heterozygous (6%) for the -175t allele. Although the number of patients analysed is small, the frequency of this allele appeared to be higher within each tribal group (2/6 Ngunis and 2/10 Sothos with FH)

compared to the controls (4/56 Ngunis and 2/40 Sothos). An overall statistically significant difference ($p < 0.05$) was observed between the presence of the rare t allele in the general black population (0.03) compared to its frequency of 0.13 in the patients diagnosed with classical or probable FH ($\chi^2 = 5.916$, 1 df, $p = 0.0149$). We furthermore detected five carriers of the $-175g \rightarrow t$ polymorphism among 40 lipid clinic patients without the FH phenotype (13%), showing an intermediate allele frequency of 0.06. This was not significantly different from the frequencies observed in the FH ($\chi^2 = 1.326$, 1 df, $p = 0.249$) or control ($\chi^2 = 1.474$, 1 df, $p = 0.224$) groups. Variant $-175g \rightarrow t$ was also detected in 1/47 DNA samples of controls from the Venda tribe studied by Ehrenborg *et al.*,²⁵ which was absent in more than 300 whites screened.²⁶

Discussion

Numerous low density lipoprotein receptor (*LDLR*) gene mutations (>600) have been identified in FH patients, but genetic data on black African populations are rare.^{19 22 23 27} A striking finding is that a 6 bp deletion predominates in a small number (5/18) of FH patients (this study)^{19 23 28} identified in the South African black population, where this lipid disorder is thought to be rare. This deletion in exon 2 removes an aspartic acid and a glycine from the first cysteine rich ligand binding repeat of *LDLR*, and impairs its transport but not lipoprotein binding in fibroblasts.²³ Frequent detection of a deleterious mutation can be the result of consanguinity, recurrent mutational events, genetic drift, founder gene effect, multiple introduction of the mutation into a population, or heterozygote advantage.

The 6 bp deletion identified originally in a homoallelic Xhosa FH homozygote,²³ and now also in a homozygous Pedi and three FH heterozygotes (Pedi and two Tswanas) on the same haplotype, have not (yet) been reported in other populations. These findings largely exclude the likelihood of a recurrent mutational event because of slipped mispairing or multiple entries of the deletion mutation into the black population. Detection of the deletion in different tribes suggests that it originated in Africa approximately 3000 years ago before tribal separation.²⁹ Although FH patients with the deletion may therefore be distantly related, family ties cannot at present explain its relatively high prevalence among black FH patients. The apparently low prevalence of FH in South African blacks and the large population size furthermore argue against a founder effect. It is, however, possible that the deletion mutation was propagated and inherited within a small group of people who later evolved separately into different African tribes. Another plausible explanation is that this deleterious deletion mutation may be associated with a selective advantage in Africa. Already in 1990 Hobbs *et al.*³⁰ noted that the presence of several founder mutations in different South African population groups^{4 31} may be indicative of a Darwinian selection that favours the hetero-

zygous state in this region of the world. Since the most likely selective agent in Africa would be infectious diseases, the finding that *LDLR* deficient mice are protected against lethal endotoxaemia and severe gram negative infections³² supports the likelihood of such an evolutionary selection mechanism conferring a survival advantage. In addition to binding and inactivating endotoxin, lipoproteins also bind certain viruses and inhibit their infectivity.³³

Although the family data presented in this study show that the $-175g \rightarrow t$ polymorphism residing in a cis acting element in the *LDLR* promoter³⁴ does not cause the FH phenotype in affected subjects, further studies are warranted to investigate the likelihood that this variant may influence disease expression. The possibility that the significantly higher frequency of the $-175g \rightarrow t$ promoter polymorphism in South African black FH patients compared to controls ($p < 0.05$) is caused by linkage disequilibrium with another downstream mutation causing the FH phenotype was excluded by haplotype studies showing that the rare t allele was associated with different *LDLR* haplotypes. This allele furthermore cosegregated with missense mutation E387K in one family. These different chromosomal backgrounds may be the result of recombination events, reflecting the age of the $-175g \rightarrow t$ variant. Compared to whites, blacks are considered older in evolutionary terms³⁵ and can therefore be expected to have accumulated variation over longer times. It is possible that the $-175g \rightarrow t$ polymorphism did not spread to other parts of the world, thereby explaining its apparent absence in whites (this study).^{18 36} The African origin of the $-175g \rightarrow t$ variant was confirmed by detection of the rare t allele at a low frequency in control DNA samples obtained from Nigerians and African-Americans.²⁶ African-Americans originated mostly from the western African coast and arrived in North America between the 16th and 19th centuries.

One Sotho proband was heterozygous for a known splicing defect in intron 3 (313+1G→A) and for the R232W mutation in exon 5. In all the patients with mutation 313+1G→A studied to date, the splicing defect is associated with a clinical picture of severe hypercholesterolaemia and early CHD.^{37 38} Patient SH had a TC concentration of 13 mmol/l, but it is uncertain whether this high level is solely because of the 313+1G→A mutation or whether there is an additional effect of the downstream R232W mutation. Family studies could not rule out the possibility of a double mutation, but showed that the splicing defect is the consequence of a de novo mutation. None of the family members of SH were hypercholesterolaemic, including his 72 year old father (LDLC 1.9 mmol/l), who was heterozygous for mutation R232W. This finding indicates that R232W does not affect *LDLR* function or, alternatively, that clinical expression of this missense mutation is altered by other genetic/environmental factors.

Although the missense mutations identified have not been characterised further, they are likely contributors to the FH phenotype in our

patient sample, since all the codon changes involve conserved amino acids and were not detected in the normal population. Screening for mutations causing FDB^{16 17 39} resulted in the identification of two silent mutations, T3540T and T3552T (data not shown), previously described in a Nigerian and African-American subject, respectively.¹⁷ Failure to identify disease related mutations in all the patients studied may be because of limitations imposed by the screening techniques used, clinical misdiagnosis of FH, or mutations in other genes causing the ADH phenotype.^{40 41}

Both the Zulu and Pedi patients clinically diagnosed with homozygous FH presented with relatively low pretreatment TC levels (<15 mmol/l) for this severe condition¹ and neither have yet suffered from CHD. The relatively mild expression of homozygous FH in these subjects largely precludes an estimation of the prevalence of heterozygous FH in the South African black population based on the prevalence of homozygous FH. Raised plasma cholesterol levels causing FH in a family frequently remain undetected until the occurrence of coronary events or clinical signs indicative of FH is observed in one or more family members. This may particularly be the case in the South African black population, as hypercholesterolaemics with lipid profiles compatible with the diagnosis of heterozygous FH frequently lack xanthomata characteristic of this condition (this study).⁴² None of the FH heterozygotes with the relatively severe 6 bp deletion in exon 2²³ presented with CHD. These findings provide evidence that FH is probably underdiagnosed in the South African black population, most likely as a consequence of altered expression of FH related mutations. This may be the result of interaction with other genetic and environmental factors, including a prudent diet.¹¹ Data provided by us and others⁴³⁻⁴⁵ therefore suggest that clinical/biochemical criteria for the diagnosis of FH need to be different by country/population and that DNA methods may assist in making a definitive disease diagnosis.

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