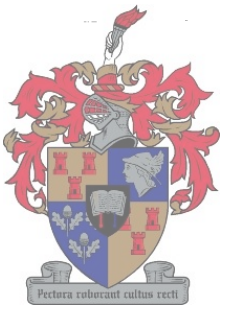
COMPARATIVE ANALYSIS OF FAMILIAL HYPERCHOLESTEROLAEMIA IN DIFFERENT POPULATIONS



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Dissertation presented for the Degree of Doctor of Philosophy at the University of Stellenbosch

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Declaration

I, the undersigned, hereby declare that the work contained in this dissertation is my own original work and that I have not previously in its entirety or in part submitted it at any university for a degree.

Date:.....11/2/2000.....

Summary

Familial hypercholesterolaemia (FH) and familial defective apolipoprotein B-100 (FDB) are relatively common disorders of lipid and lipoprotein metabolism caused by mutations in the low density lipoprotein receptor (LDLR) and apolipoprotein B (apo B) genes, respectively. DNA analyses at these loci were performed in 132 molecularly-uncharacterised South African, 11 Costa Rican and 13 New Zealand subjects with clinical features of heterozygous FH. Mutation R3500Q causing FDB was identified in a relatively large proportion (~30%) of the New Zealand patients. LDLR gene defects were identified in 4 Costa Rican and 6 New Zealand FH patients. Sixty-five different LDLR gene mutations were identified in South African hypercholesterolaemics, revealing ten founder-type mutations.

Haplotype analysis at the LDLR and apo B loci excluded the likelihood that mutations in these two genes underlie the FH phenotype in one of the New Zealand families. The apparently autosomal dominant hypercholesterolaemia (ADH) in this family could also not be linked to a newly identified gene locus, designated FH3. Analysis of the New Zealand study cohort, although small, demonstrated both mutational and locus heterogeneity in ADH.

Analysis was also extended to include subjects from the various ethnic groups within South Africa. The high prevalence of FH in Afrikaners of European descent is in striking contrast to the reported virtual absence of this lipid disorder in the Black South African population. In addition to three previously-described Afrikaner founder mutations (D154N, D206E and V408M), four minor founder mutations, D200G, S285L,

C356Y and G361V, were identified in 12 Afrikaner families. Surprisingly, a 6-bp deletion in exon 2 of the LDLR gene was detected at a relatively high frequency (28%) in Black FH patients. This finding, as well as clinical correlations performed in the patients, suggests that the expression of FH mutations in the Black population may be altered due to interaction with other genetic and/or environmental factors, therefore leading to underdiagnosis of the disease. Common LDLR gene mutations have also been described in South African Indians (P664L) and Jews (del 197), most likely as a consequence of multiple introductions of defective genes into these relatively isolated communities. Caucasoid admixture was recognised as a major factor contributing to the FH phenotype in the indigenous South African population of mixed ancestry from the Western Cape, where six founder-type mutations account for the disease in 22% of cases. The high prevalence of specific LDLR gene mutations in different population groups facilitates an improved diagnostic service for FH in South Africa.

Opsomming

Familiële hipercholesterolemie (FH) en familiële defektiewe apolipoproteïen B-100 (FDB) is relatief algemene afwykings in lipied en lipoproteïen metabolisme wat onderskeidelik veroorsaak word deur mutasies in die lae digtheids lipoproteïen reseptor (LDLR) en apolipoproteïen B-100 (apo B) gene. Molekulêre DNS analise van hierdie lokusse is uitgevoer in 132 Suid Afrikaanse, 11 Costa Rikaanse en 13 New Zealandse pasiënte waar die geen mutasies onderliggend aan die kliniese beeld van heterosigotiese FH onbekend was. Mutasie R3500Q wat FDB veroorsaak was in 'n relatief groot aantal van die New Zealandse pasiënte (~30%) teenwoordig. LDLR geen defekte is in 4 Costa Rikaanse en 6 New Zealandse FH pasiënte geïdentifiseer. Vyf en sestig verskillende LDLR geen mutasies is aangetoon in die Suid Afrikaanse populasie waarvan tien stigter-geen mutasies is.

Haplotipe analise van die LDLR en apo B lokusse het die moontlikheid uitgesluit dat mutasies in hierdie twee gene verantwoordelik is vir die FH fenotipe in een van die New Zealandse families. Die waarskynlik outosomaal dominante hipercholesterolemie (ODH) in hierdie familie kon ook nie toegeskryf word aan 'n nuwe geïdentifiseerde geen lokus genaamd FH3 nie. Analise van die New Zealandse studie paneel het dus beide mutasie en lokus heterogeniteit in ODH gedemonstreer.

Analise was uitgebrei deur die toevoeging van individue van verskeie etniese groepe van Suid-Afrika. Die hoë voorkoms van FH in Afrikaners van Europese afkoms is in opvallende kontras met die voorheen vermeende feitlike afwesigheid van hierdie lipied afwyking in die Swart Suid-Afrikaanse populasie. Afgesien van drie bekende Afrikaner

stigter mutasies (D154N, D206E en V408M), is nog vier relatief algemene mutasies, D200G, S285L, C356Y en G361V, geïdentifiseer in 12 Afrikaner families. 'n Onverwagse bevinding was die opsporing van 'n 6-bp delesie in ekson 2 van die LDLR geen teen 'n relatief hoë frekwensie (28%) in Swart FH pasiënte. Hierdie bevinding, sowel as kliniese korrelasies wat in hierdie groep pasiënte uitgevoer is, impliseer dat FH moontlik ondergediagnoseer word in die Swart populasie weens interaksie van defektiewe LDLR gene met ander genetiese en/of omgewingsfaktore. Algemene LDLR geen mutasies is ook beskryf in Suid Afrikaanse Indiërs (P664L) en Jode (del 197), heel waarskynlik as 'n gevolg van veelvuldige oordrag van defektiewe gene in hierdie relatief geïsoleerde gemeenskappe. Kaukasiër vermenging is herken as 'n belangrike faktor onderliggend aan die FH fenotipe in die inheemse Wes-Kaapse kleurling populasie van Suid-Afrika, waar ses stigter-tipe mutasies verantwoordelik is vir die siekte in 22% van gevalle. Die hoë voorkoms van spesifieke LDLR geen mutasies in verskillende populasie groepe maak populasie-gerigte DNA diagnose van FH moontlik in Suid Afrika.

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Abbreviations

µg	microgram
µl	microlitre
µM	micromoles per litre
ADH	autosomal dominant hypercholesterolaemia
apo B	apolipoprotein B-100
APS	ammonium persulfate
bp	base pair/s
cDNA	complementary deoxyribonucleic acid
CHD	coronary heart disease
CVD	cardiovascular disease
del	deletion
DGGE	denaturing gradient gel electrophoresis
dH_2O	deionized, distilled water
DNA	deoxyribonucleic acid
dNTP	deoxynucleoside triphosphate
EDTA	ethylenediaminetetra-acetic acid
EGF	epidermal growth factor
FCHL	familial combined hyperlipidaemia
FD	familial dysbetalipoproteinaemia
FDB	familial defective apolipoprotein B-100
FH	familial hypercholesterolaemia
FHT	familial hypertriglyceridaemia
FP	footprinting
HDL	high density lipoprotein
HEX-SSCP	heteroduplex-single strand conformation polymorphism
HMG CoA	hydroxymethylglutaryl coenzyme A
kb	kilobase
LDL	low density lipoprotein

LDLR	low density lipoprotein receptor
Lp(a)	lipoprotein(a)
MEDPED	Make Early Diagnosis to Prevent Early Deaths in MEDical PEDigrees
MI	myocardial infarction
ml	millilitre
mM	millimoles per litre
mmol/l	millimoles per litre
pmol	picomole
PCR	polymerase chain reaction
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
SDS	sodium dodecyl sulphate
SP	signal peptide marker
SSCP	single-strand conformation polymorphism
SRE	sterol regulatory element
TEMED	N,N,N',N'-tetramethylethylenediamine
TAE	Tris-acetic acid-EDTA
TBE	Tris-borate-EDTA (pH 8.3)
TC	total cholesterol
TG	triglyceride
$(TG)_n$	dinucleotide TG repeats
VLDL	very low density lipopotein
VNTR	variable number of tandem repeats
XMTA	xanthomata

Contents

Chapter 1

Introduction

Coronary Heart Disease (CHD) is one of the leading causes of death in Westernised countries, where it accounts for approximately 25% of all mortality. Elevated plasma cholesterol concentrations leading to atherosclerosis affect approximately 4.8 million South Africans and place them high at risk of developing CHD. Although CHD remains uncommon in urban blacks (Walker 1999), it has been noted that the prevalence of this condition is increasing in this population in South Africa.

Dyslipidaemia is caused by a combination of heritable and environmental factors. Primary hyperlipidaemias include (i) familial hypercholesterolaemia (FH), (ii) familial defective apolipoprotein B100 (FDB), (iii) familial combined hyperlipidaemia (FCHL), (iv) polygenic hyperlipidaemia, (v) familial hypertriglyceridaemia (FHT) and (vi) familial dysbetalipoproteinaemia (FD). These genetic causes of dyslipidaemia are classified in categories of Fredrickson (Fredrickson et al. 1967) type IIa (i, ii, iv), types IIa, IIb and IV (iii), types IV and V (v) and type III hyperlipoproteinaemias (vi).

Lipoprotein abnormalities are a result of disorders of cholesterol metabolism, including low density lipoproteins (LDL), very low density lipoproteins (VLDL), remnants and chylomicrons. Secondary dyslipidaemia is associated with other conditions, such as diabetes mellitus, hypothyroidism, obesity and alcohol abuse.

1.1 Cholesterol metabolism

Cholesterol plays an important role in the production of bile, steroid hormones (cortisone) and sex hormones (androgen/oestrogen), and is vital for the intactness of cell membranes. The synthesis of cholesterol is mediated by hydroxymethylglutaryl coenzyme A (HMG CoA) reductase from acetyl coenzyme A (Acetyl-CoA), via mevalonic acid, squalene, and other intermediates.

Cholesterol is transported in lipoproteins (protein/lipid macromolecules) that circulate in the bloodstream. Four types of lipoprotein abnormalities are frequently found in the population and are present in 50-80% of myocardial infarction (MI) survivors (Breslow 1992). These lipoprotein defects are: (i) increased chylomicron remnant- and intermediate-density lipoprotein (IDL) concentrations; (ii) increased very-low-density lipoprotein (VLDL) levels and decreased high-density lipoprotein (HDL)-cholesterol levels; (iii) increased low-density lipoprotein (LDL)-cholesterol levels and (iv) increased Lp(a) levels.

Chylomicrons (secreted by the intestine) and VLDL (secreted by the liver) are the two triglyceride (TG)-rich lipoproteins. The chylomicrons carry the exogenous TG. The exogenous (or dietary) component of cholesterol comes from saturated fats which are converted by the body into LDL cholesterol or LDL. The precursor of LDL, VLDL, carries endogenous TG. The endogenous (metabolic) component of cholesterol comes from the synthesis of cholesterol in the cells of the body, particularly the liver cell or hepatocyte. The LDL deposits cholesterol from the blood in the arteries (via a protein carrier, apoprotein B) and the HDL transports excess cholesterol (via a protein carrier, apoprotein A) from the arteries and returns it to the liver where it is broken down for removal from the body. Lp(a) is a modified form of LDL and is a risk factor for the development of CHD (Utermann 1989).

The LDLR is a transmembrane glycoprotein that binds LDL and mediates its uptake into the liver cell or hepatocyte. In the absence of functional receptors, LDL's remain in the blood plasma which cause deposits to form inside blood vessels. The deposits are formed through an oxidation interaction between the LDL and free radicals on the inside wall of the artery. These deposits causing atherosclerosis are called plaques. The accumulation of cholesterol inside the hepatocyte down-regulates the expression of the LDLR gene.

In hyperlipidaemics, the risk of developing CHD can greatly be reduced by the use of appropriate drugs (e.g. cholestyramine, HMG-CoA reductase inhibitors) and by eliminating or modifying additional risk factors. Preventive measures include a cholesterol-free diet, avoiding stress, weight control, physical exercise, regular evaluation of hypertension, and cessation of smoking. If the liver enzyme HMG CoA reductase is blocked (by statins), cholesterol synthesis ceases and the LDLR is stimulated to siphon more cholesterol into the cell, thus lowering the serum LDL. Costly procedures such as LDL apheresis and liver transplantation may prolong life of homozygous FH patients.

1.2 Autosomal dominant hypercholesterolaemia (ADH)

Several genetic conditions have been identified that affect lipid levels. The clinical manifestations of severely elevated LDL cholesterol concentration are defined as two relatively common genetic disorders, namely familial hypercholesterolaemia (FH) (Goldstein et al. 1995) and familial defective apolipoprotein B-100 (FDB) (Innerarity et al. 1990). FH is caused by mutations in the LDLR gene (Hobbs et al. 1992) and FDB by mutations in the apoB gene (Soria et al. 1989). Recently, a third major locus for ADH, designated FH3, was mapped to chromosome 1p34.1-32 (Varret et al. 1999/Appendix 3, Hunt et al. 1999). This genome-wide gene search performed by Varret et al. (1999)

(Appendix 3) also provided evidence of a fourth locus underlying the FH phenotype. The genetic heterogeneity of FH was confirmed by Haddad et al. (1999), who excluded the involvement of mutations in the LDLR- and apoB genes in a clinically diagnosed FH family.

Familial combined hyperlipidaemia (FCHL) is a common genetic disorder of unknown cause that accounts for up to 10% of patients with myocardial infarction (MI) (Brown and Goldstein 1991). FCHL affected patients may have elevated LDL cholesterol levels, elevated VLDL triglyceride levels or both within the same family. Pajukanta et al. (1998) localised a locus associated with FCHL on human chromosome 1q21-q23 in families from a Finnish isolate. The hyperlipidaemia gene (*Hyplip1*) for combined hyperlipidaemia was furthermore mapped to chromosome 3 in a mutant mouse strain, which is syntenic to human chromosome 1q21-q23 (Castellani et al. 1998). Additional putative FCHL loci have recently been reported in Finnish families with the TG, TC or apoB traits, after a genome-wide screen (Pajukanta et al. 1999).

There is now also evidence of a new lipid disorder, an autosomal recessive form of FH (Zuliani et al. 1999). It was suggested that the disease phenotype in two Sardinain families may be due to marked reduction of *in vivo* LDL catabolism, caused by a selective reduction in hepatic LDL uptake.

1.2.1 Familial hypercholesterolaemia (FH)

FH is a co-dominantly inherited lipid disorder with a heterozygote frequency of approximately 1 in 500 individuals in most populations. The homozygous form is rare, occurring in approximately 1 in a million individuals (Goldstein et al. 1995).

FH heterozygotes express half the normal LDLR activity, which may result in a twofold increase in the plasma concentrations of LDL cholesterol levels (above the 95th

percentile for age and gender), the presence of visible LDL-derived cholesterol deposits in the tendons of the hands and feet (tendon xanthomas) and premature CHD (Goldstein et al. 1995). Homozygotes express little or no LDLR activity and therefore the plasma cholesterol levels are higher (15.0 mmol/l or above) and the generalised atherosclerosis and cardiovascular disease (CVD) more severe. In FH homozygotes, this phenotype is associated with death in childhood. In heterozygous FH patients, the disease may appear in the early 20's and, without treatment, it is quite likely that the patient will die early from a heart attack. In men this occurs between the ages 35 to 55 years and in women by ages 55 to 75 years, since women are probably protected by oestrogen (Pyorala et al. 1994).

Mutations in the LDLR gene have been extensively studied as a cause of the clinical manifestations of ADH. This gene, located on the short arm of chromosome 19p13.1-p13.3, is composed of 18 exons coding for a membrane-bound glycoprotein of 839 amino acids (Yamamoto et al. 1984, Südhof et al. 1985). The mature LDLR consists of five distinct functional domains: ligand binding, exons 2-6; epidermal growth factor (EGF) precursor homology, exons 7-14; carbohydrate side chains (O-linked sugars), exon 15; membrane spanning, exons 16-17 and cytoplasmic, exons 17-18; with its N-terminal signal sequence (21 hydrophobic amino acids) on the outside of the plasma membrane and the C-terminal sequence inside the cell cytoplasm (Russell et al. 1984). The promoter (5'-flanking) region, located within the first 200 bp upstream of the initiation codon of exon 1, consists of *cis*-acting DNA sequence (mRNA initiation sites, two TATA boxes; three closely spaced 16 bp direct repeats; two sterol regulatory elements) that control LDLR gene transcription (Südhof et al. 1987). The 16 bp repeats are responsible for binding of several transcription factors. Repeats 1 and 3 contain Sp1-binding sites, a *trans*-acting transcription factor, which are responsible for basal transcription of the LDLR gene in the presence and absence of sterols (Dawson et al. 1988). Repeat 2, designated the sterol regulatory element (SRE 1), interacts

with essential transcription binding proteins (SREBP-1) to induce high-level expression of the LDLR gene (Yokoyama et al. 1993, Oliner et al. 1996, Streicher et al. 1996). SRE 1 is responsible for sterol-mediated repression of the gene when cellular cholesterol levels are high (Smith et al. 1990, Briggs et al. 1993). Footprinting 1 (FP1) and footprinting 2 (FP2), two *cis*-acting regulatory elements, are essential for maximal induction of transcription (Mehta et al. 1996). Sterols, cytokines, growth factors and hormones are additional substances that may influence regulation of transcription of the LDLR gene (Dawson et al. 1988, Mazzone et al. 1989, Rudling et al. 1992, Stopeck et al. 1993).

Several DNA polymorphisms have been identified in the LDLR gene that can be used to follow the inheritance of the defective gene in FH families (Hobbs et al. 1992). Genetic markers provide the necessary tools for the identification of different or recurrent FH mutations, to study the origin of a common mutation (a particular mutation is likely to be associated with a specific haplotype) and for co-segregation analysis as a basis for (prenatal) DNA diagnosis of FH where the disease-causing mutation has not been identified. Four intragenic markers defined by the restriction enzymes *Sma*I (exon 7), *Stu*I (exon 8), *Ava*II (exon 13) and *Nco*I (exon 18) and two highly informative microsatellite markers, D19S221 and D19S394 (Haddad et al. 1997), located 1.3 Mb centromeric and 250 kb telomeric to the LDLR gene respectively, are frequently used for this purpose in population studies.

To date, more than 600 different mutations have been described in the LDLR gene world wide (http://www.ucl.ac.uk/fh and http://www.umd.necker.fr.). Point mutations, small deletions and insertions are responsible for the majority of FH cases. About 15 % of all point mutations are situated at splice junctions (Krawczak et al. 1992), and similar findings have recently been described in FH patients (Peeters et al. 1999). Large gene alterations have been detected in the minority of cases, mostly by Southern blot analysis prior to the availability of polymerase chain reaction (PCR)-based assays. The mutations have been

grouped into five classes based on their phenotypic effects on LDLR function. Class 1 mutants fail to produce receptors (null alleles). Class 2 mutants produce receptors, but do not transport them from the endoplasmic reticulum (ER) to the Golgi apparatus (transport defective alleles). Class 3 mutant receptors reach the cell surface, but fail to bind LDL (binding defective alleles). Class 4 mutant receptors reach the cell surface and bind LDL, but fail to cluster in coated pits and cannot internalise LDL (internalisation defective alleles). Class 5 mutant receptors fail to discharge the ligand in the endosome and cannot recycle to the cell surface (recycling defective alleles).

Most individuals with heterozygous FH are not diagnosed with the disease and are therefore not treated adequately to prevent or delay the development of CHD. This situation led to the initiation of an international project aimed at Making Early Diagnosis to Prevent Early Deaths in MEDical PEDigrees (MEDPED). MEDPED is based on a family screening approach to identify and treat affected FH individuals (Williams et al. 1993). FDB patients are also included in the MEDPED project, since FH and FDB share similar clinical and biochemical characteristics. The MEDPED initiative may in future elect to include other familial lipid disorders such as type III hyperlipidaemia, familial combined hyperlipidaemia (FCHL) or polygenic hypercholesterolaemia, and can also be applied to other common genetic diseases (Kotze and Callis 1999).

1.2.2 Familial defective apolipoprotein B-100 (FDB)

FDB occurs as a consequence of LDL particles which bind poorly to the LDLR, because of mutations in the binding area of the apo B gene. This gene is the main constituent peptide of LDL and serves as the ligand for receptor-mediated uptake of LDL (Brown and Goldstein 1986). The apo B gene spans 43 kb on chromosome 2p23-24 (Knott et al. 1985) and has 29 exons and 28 introns (Blackhart et al. 1986). The most frequent mutation is a glutamine to

arginine substitution at codon 3500 (R3500Q) in exon 26 of the gene (Soria et al. 1989). Several studies have indicated that FDB is associated with a relatively mild disease, comparable to polygenic hypercholesterolaemia (Tybjerg-Hansen and Humphries 1992, Miserez and Keller 1995) or receptor-defective FH (Kotze et al. 1994). The clinical phenotype of FDB homozygotes is less severe than those of FH homozygotes (Schaefer et al. 1997).

This dominantly inherited genetic disorder occurs in approximately 1 in 500 individuals in most European countries. Higher frequencies have been reported in Switzerland (Miserez et al. 1994) and Belgium (Kotze et al. 1994), and lower frequencies south of the Alpes, in Russia, in Scandinavia, and in Denmark (Hansen 1998). FDB is rare in the South African population, most likely as a result of under representation of mutation R3500Q in South African settler populations (Rubinsztein et al. 1995). Previous studies have indicated that the common FDB 3500 mutation originated in Europe, prior to racial diversity (Ludwig and McCarthy 1990).

1.3 Common LDLR gene mutations in different populations

FH tends to occur more frequently in certain populations of the world, such as the Afrikaners in South Africa, the French Canadians, the Finnish, the Lebanese Christian Arabs, and Lithuanian Jews (Table 1). This phenomenon is probably a consequence of the establishment of these populations by a few original founders who carried only a small fraction of the total genetic variation of the parental population (Mayr 1963, Davignon and Roy 1993).

Table 1. Populations where FH is prevalent due to founder LDLR gene mutations.

Country	Population	Prevalence	Mutation	Allele designation	Ref*
South Africa	Afrikaners	1 in 70	D206E	FH Afrikaner-1	1, 2
			V408M	FH Afrikaner-2	1, 2
			D154N	FH Afrikaner-3	2
	Jews	1 in 100	197delGGT	FH Lithuanian	3
	Indians	1 in 100	P664L	FH Gujerat	4
Canada (Quebec-Province)	French- Canadians	1 in 270	>15-kb del	FH French Canadian-1	5
			5-kb del	FH French Canadian-5	6
			W66G	FH French Canadian-4	7
			E207K	FH French Canadian-3	7
			C646Y	FH French Canadian-2	7
			Y468X	-	8
Finland (North Karelia)	Finnish	1 in 441	9-kb del	FH Helsinki	9
			7-bp del at 925	FH North Karelia	10
			G823D	FH Turku	11
			L380H	FH Pori	11
Israel	Christian Lebanese	1 in 100	C660X	FH Lebanese	12
	Ashkenazi Jews	1 in 80	197delGGT	FH Lithuanian	3
	Druze		Y167X	-	13
	Sephardic Jews		D147H	-	14

*References: (1) Leitersdorf et al. 1989, (2) Kotze et al. 1989, 1991, (3) Meiner et al. 1991, (4) Kotze et al. 1997, (5) Hobbs et al. 1987, (6) Ma et al. 1989, (7) Leitersdorf et al. 1990, (8) Simard et al. 1994, (9) Koivisto et al. 1992 (10) Aalto-Setälä et al. 1989, (11) Koivisto et al. 1995, (12) Lehrman et al. 1987, (13) Landsberger et al. 1992, (14) Leitersdorf et al. 1993

The relatively high frequency of a disease in some populations because of a founder effect and/or genetic drift, following the introduction of a deleterious mutation in the gene pool, may be explained mainly through migration and geographical or cultural isolation (Zlotogora 1994).

A set of alleles at polymorphic sites within a given gene, that are in linkage disequilibrium with a mutation, may represent the founder haplotype (Labuda et al. 1997). This information on the chromosomal background of a given mutation can be used to trace back a genetic alteration to one common ancestor whilst the spread of specific FH alleles can also be followed in different parts of the world. Haplotype analysis can also provide information on the possible independent occurrence of a specific mutation in patients from different populations. The identification of founder mutations in the LDLR gene has been a

helpful tool in the implementation of genetic screening programs in specific population groups (Leitersdorf et al. 1989, 1990, Koivisto et al. 1992, Kotze et al. 1995).

South Africa

Founder-related LDLR gene defects underlie the high prevalence of FH in several South African ethnic groups, including in the Afrikaners (Caucasians), Jews (Ashkenazi) and Indians. This is largely due to the early and/or recurrent introduction of specific mutations into relatively isolated communities and subsequent population expansion (Kotze et al. 1991, Meiner et al. 1991, Rubinsztein et al. 1994). An apparently low FH frequency has been reported in the South African Black population, originating from central Africa.

In the South African Afrikaner population three LDLR gene mutations, D154N, D206E and V408M, account for ~90% of FH in affected individuals (Leitersdorf et al. 1989, Kotze et al. 1989, 1991). Mutation V408M that is responsible for ~15% of FH in Afrikaners was also detected by Defesche et al. (1993) in the Netherlands and by Schuster et al. (1993) in Germany. These findings reflect the fact that Afrikaners originated from about 2 000 settlers who immigrated from Holland, Germany and France in the 17th and 18th centuries. Approximately 1 million of the roughly 2.5 million Afrikaners who were founded by one shipload in 1652 still have the surnames of 20 original settlers (http://helix.biology.mcmaster.ca/3j3/3j3.founder). The first British immigrants arrived in South Africa around 1820, forcing the Dutch settlers to the north (Transvaal) (Botha and Beighton 1983). These groups were isolated for a long time from other populations, due to cultural and religious differences. Mutations D154N and D206E occur in approximately 10% and 65% of FH patients in the Afrikaner population, respectively (Kotze et al. 1991, Graadt van Roggen et al. 1991). These common Afrikaner mutations identified in exon 4 were found in the Netherlands in FH patients of British origin, but were not identified in the Dutch

population (Defesche et al. 1993). Mutation D206E was also identified in FH patients from London (Gudnason et al. 1993). It is therefore highly likely that these mutations were introduced into South Africa by British immigrants.

The three Afrikaner founder mutations represent 15% of disease-related mutations in the Coloured population of South Africa, a people of mixed ancestry (San, Khoi, African Negro, Madagascar, Javanese and European origin) (Loubser et al. 1999). Caucasoid admixture therefore contributes significantly to the FH phenotype in this indigenous South African population. A 2.5 kb deletion of exons 7 and 8 is also relatively common (~10%) in South Africans of mixed ancestry (Henderson et al. 1988). A study performed by Peeters et al. (1997) excluded the likelihood that this deletion is the same one described by Top et al. (1990) in the Dutch population.

The increased prevalence of FH in South African Jews and Indians appears to be a consequence of recurrent introduction of specific LDLR gene mutations into these relatively isolated communities. The predominant FH Lithuania mutation in the Ashkenazi Jews entails a 3-bp deletion of codon 197 and was also identified in the majority of South African FH-Jews (Meiner et al. 1991). This mutation may be responsible for FH in up to 80% of South African Jews. Lithuania immigrants who came to South Africa between 1880 and 1910 gave rise to most of the inhabitants of the South African Ashkenazi population.

Mutation P664L that is prevalent in the West of India, the Gujerat Province, is the most common mutation (~50%) among South African Indians (Kotze et al. 1997). Their ancestors came to South Africa as traders under British auspices between 1860 and 1911. Most were from areas in the Gujerat province and the other major group came from Kathiowar on the Arabian Sea. South African Indians are of diverse cultural and religious origin; 65% Hindus, 21% Muslims and 7.5% Christians (Rubinsztein et al. 1994).

The majority of mutations identified to date in Africans are those detected in the South African Black population (Hobbs et al. 1992, Varret et al. 1998/Appendix 2). In this study it is shown that a 6-bp deletion in exon 2 of the LDLR gene (Leitersdorf et al. 1988) predominates in the South African Black population.

Canada

The Canadians, with the exception of French-Canadians and the indigenous Indians, constitute a heterogeneous population of mainly British, Irish and Dutch descent. The French Canadian population descended from approximately 7 000 original settlers who emigrated from northeastern and western France to eastern Canada in the 17th and 18th centuries (Laberge 1966). This population remained genetically isolated from English influences due to social and geographic factors. The FH prevalence varies between different regions among French Canadians: Bas St-Laurent/Gaspésie (1 in 167), Côte-Nord in Northeastern Quebec (1 in 81) and SLSJ (1 in 122) (Moorjani et al. 1989). Eleven LDLR gene mutations account for more than 90% of FH cases in the French-Canadian population (Minnich et al. 1995, Vohl et al. 1997, Couture et al. 1999). Two deletions represent the most common mutations. A >15-kb deletion of the promoter and exon 1 resulting in a null allele (Hobbs et al. 1987), shows the highest frequency (71.2%) in the Bas St-Laurent/Gaspésie region, while a 5-kb deletion of exons 2 and 3 (Ma et al. 1989) represent 2% of FH cases. Missense mutation W66G had the highest frequency (59.2%) in the Saguenay-Lac-St-Jean/Côte-Nord region. Mutations E207K (3%), C646Y (6%) (Leitersdorf et al. 1990) and Y468X also occur frequently in this population (Simard et al. 1994). The large deletion was also identified in France (Fumeron et al. 1992), and ancestors of individuals with this so-called French Canadian deletion were traced back from the same region in northeast of Montreal, Canada, called Kamouraska (Jompe et al. 1988).

Finland

Finns emigrated in the 16th and 17th century to Värmlandin, the central part of Sweden and then further west to the Eastern part of Norway (Opsahl and Winge 1990). The highest density of FH patients in the Polvijärvi region of North Karelia represent a FH prevalence of 1 in 143 (Vuorio et al. 1997). A 9.5-kb deletion from intron 15 to exon 18 and a 7-bp deletion (CCCATCA) at 925 in exon 6, are very common mutations in the Southeast and Midwest of Finland, respectively. Together these mutations account for approximately 90% of FH cases in Finland (Aalto-Setälä et al. 1989, Koivisto et al. 1993). The FH-Helsinki mutation was also identified in Norway probably as a consequence of an independent mutational event (Rodningen et al. 1992). Two missence mutations, G823D and L380H, are responsible for FH in approximately 8% of the remaining cases (Koivisto et al. 1995). Founder effects in villages with relative homogeneity within specific rural communities are thought to underline the high frequency of FH in certain areas in Finland (Levanlinna 1972, Norio et al. 1973).

Israel

Several founder LDLR mutations were identified in Israel, each in a defined group: Christian-Arabs, Ashkenazi, Sephardic Jews and Druze.

The FH Lithuania mutation, a 3-bp deletion in exon 4 (Gly 197) of the LDLR gene, is predominantly present in Ashkenazi Jews in Israel. Lithuanian Jews have immigrated to countries all over the world, which explains the world wide distribution of the Lithuanian mutation. The high incidence of this mutation among South African Jews (Meiner et al. 1991) is probably due to significant immigration of the Lithuanian Jews to South Africa.

FH among Christian Lebanese is mainly caused by a mutation in exon 14 that creates a *Hinf* I restriction site (Lehrman et al. 1987). This mutation produces a premature termination codon found to be responsible for FH in several Lebanese homozygous cases. The adult Christian-Arab population, living in the Galilee region of northern Israel, consists mainly of Lebanese who have immigrated over the last two centuries. Genetic isolation in a predominantly Muslim environment and a high degree of consanguinity contributed to the high frequency of this mutation in Christian Lebanese (Lehrman et al. 1987). The Lebanese mutation was also identified among Brazilian FH patients (Figueredo et al. 1992), most likely as a consequence of an independent mutational event.

Mutation Y167X in exon 4 is responsible for the majority of FH cases in a small Middle Eastern Islamic sect, in two distinct Druze villages, Majdal Shams and Ein Kuniye, from the Golan Height in Northern Israel (Landsberger et al. 1992). The Druze community remained isolated because of religious restrictions and have the highest prevalence of consanguineous marriages in Israel (Freundlich and Hino 1984).

Another mutation in exon 4 of the LDLR gene (D147H) causes FH in ~10% of the Jewish Sephardic population originating from Safed in Northern Israel (Leitersdorf et al. 1993, Reshef et al. 1996).

The geographical distribution of founder mutations, together with other relatively common LDLR gene mutations identified in different populations in the world, is illustrated in fig. 1. It is unlikely that a founder effect would occur and be maintained in heterogeneous populations, such as in North America and Europe. These populations have a plethora of LDLR gene mutations, which are mostly restricted to single families. Interestingly, in some European populations increased frequencies of specific LDLR gene mutations have been reported.

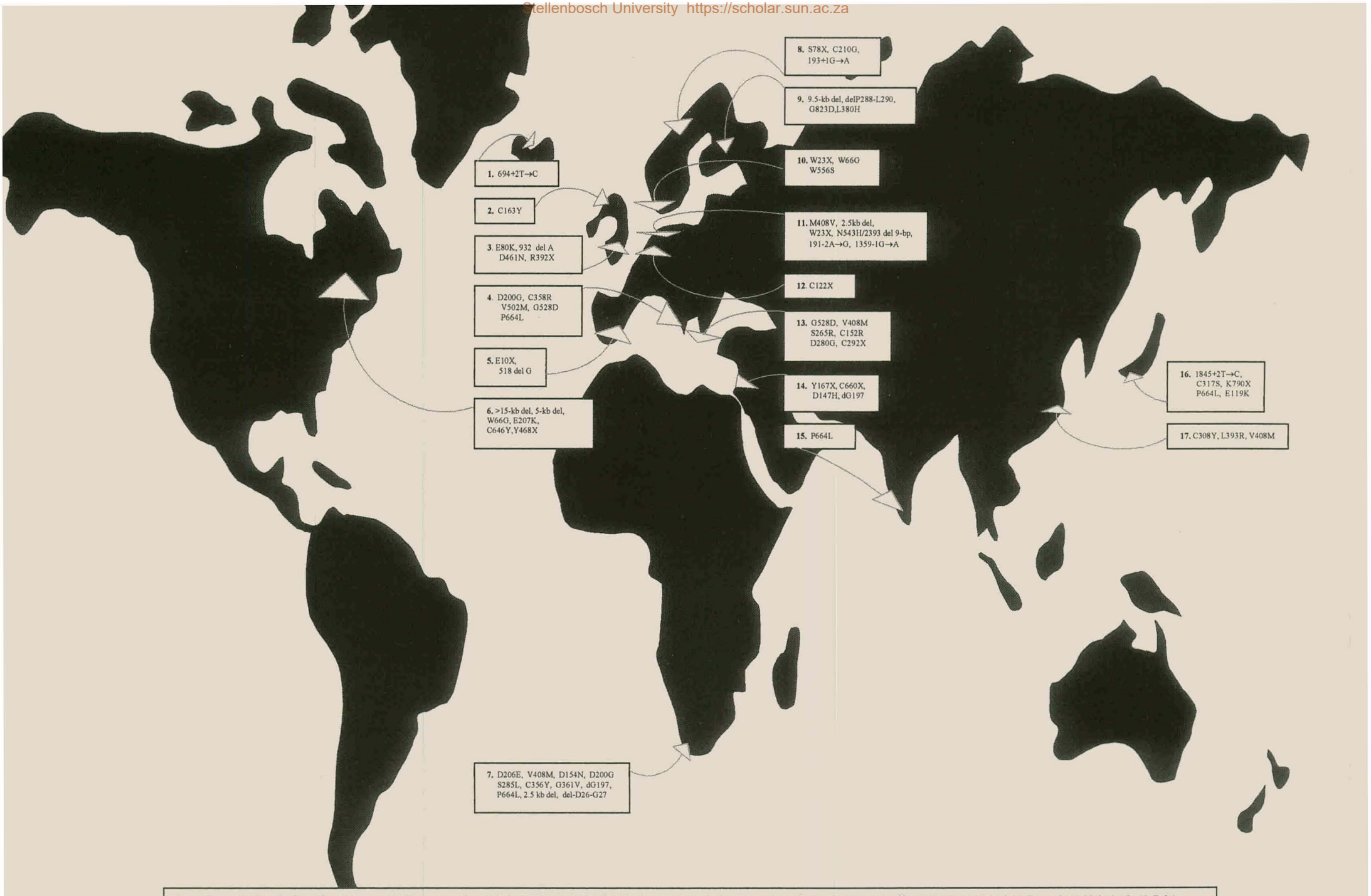

Fig. 1. Geographical distribution of founder/common LDLR gene mutations: 1. Iceland, 2. Scotland, 3. United Kingdom, 4. Italy, 5. Spain, 6. French Canadian (Quebec), 7. South Africa, 8. Norway, 9. Finland, 10. Denmark, 11. Netherlands, 12. Belgium, 13. Greece, 14. Israel, 15. India, 16. Japan, 17. China (Hong Kong).

United Kingdom

The United Kingdom harbours a genetically heterogeneous population with regard to racial and ethnic origin, and therefore the presence of a founder mutation was considered unlikely (Day et al. 1997a). However, mutation E80K (Gudnason et al. 1993) detected in Manchester, 932del A and D461N in Northern Ireland (Ward et al. 1995) and R392X (Day et al. 1997b) in Southampton, occur at frequencies of 12%, 8-9% and 10%, respectively. Haplotype studies revealed that mutation E80K arose from a single ancestor less than 1000 years ago (Wenham et al. 1998). This regional variation in the frequency of mutations might be due to population expansion over the last 100-200 years, with the effect being diluted by migration (Humphries et al. 1997).

Spain

Although the Spanish population is a heterogenous population with a broad spectrum of mutations in the LDLR gene (Cenarro et al. 1998), mutations E10X (exon 2) and 518delG (exon 4) were found to be responsible for 20% and 13.3% of FH cases, respectively (Cenarro et al. 1996). Most of these FH subjects of Spanish origin were from the Aragón region. Haplotype analysis revealed that both mutations probably originated from a common ancestor.

Belgium

Mutation C122X accounts for 16% of FH-cases in southern Belgium, but is less frequent in Northern Belgium (Descamps et al. 1997). Haplotype analysis revealed the same chromosomal background in all the families with mutation C122X, but extensive genealogical studies failed to reveal a common ancestor.

Italian

A spectrum of mutations identified in FH homozygous Italian patients confirmed the allelic heterogeneity of FH in this population. Mutations D200G, C358R, V502M, G528D and P664L were identified in 3 or more unrelated FH cases. Each mutation has been associated with the same haplotype (Bertolini et al. 1999).

Greece

Six LDLR gene mutations were identified in 60% of Greek FH heterozygotes. These mutations, G528D (22.7%), V408M (14.7%), S265R (11.3%), C152R (5.3%), D280G (3.3%) and C292X (2.7%), represent a common ancestor for each mutation, which is consistent with the geographical distribution of FH families with these mutations throughout the country. Estimation of the number of generations from a common ancestor, indicates that these mutations arose in Greece within the last 1000 years (Traeger-Synodinos et al. 1998). Mutation V408M was detected on a different haplotype than that reported for Afrikaners implying a recurrent mutational event at the CpG hotspot (Schuster et al. 1993).

Denmark

Mutations W23X (15%), W66G (15%) and W556S (12%) account for a relatively large proportion of FH cases in Denmark (Jensen et al. 1997). Haplotypes identical in families with the W23X and W66X mutations, respectively, suggesting that the patients probably had common ancestors. The high prevalence of these mutations might be due to their introduction in Denmark in small isolated tribes before the whole country was populated (Jensen et al. 1996).

Netherlands

The Afrikaner founder mutation V408M also occurs at an increased frequency (1.5%) in the Dutch FH population. This mutation probably originated in the Netherlands and was introduced into South Africa by a Dutch settler in the 17th century (Defesche et al. 1993). Four LDLR gene mutations were associated with strong geographical preferences in the Netherlands: W23X (exon 2) is predominantly found in the Northeast, the combined defect N543H (exon 11)/2393 del 9-bp (exon 17) in the Northwest, the 191-2 (A→G) (intron 2) in the southwest and 1359-1 (G→A) (intron 9) in the midsouth (Defesche and Kastelein 1998).

Scotland

A geographical preference of LDLR gene mutation C163Y (8.8%) has been reported in the Glasgow area, west of Scotland. The same D19S394 allelic size co-segregated with the FH phenotype in five of the seven probands with this mutation. This finding is compatible with the mutation having occurred relatively recently on a chromosome of that allelic size, with the allelic association being maintained because of the small genetic distance (Lee et al. 1998).

Norway

Three founder-type mutations underlying FH, S78X (8%), G→A at 193+1 (25%) and C210G (8%) (Leren et al. 1994, Tonstad et al. 1995, Sundvold et al. 1996), predominate in Norway. Mutation S78X occurred on the same chromosomal background in all five families studied by Leren et al. (1994). The splice mutation was associated with an identical haplotype in 16 of 20 families analysed.

Iceland

In Iceland the presence of a founder mutation has been reported in the western part of the country. Mutation 694+2T→C in intron 4 is present in approximately 60% of index cases and in half of these cases a common ancestor could be traced (Gudnason et al. 1997). The frequency of FH in Iceland has not been established, but is presumed to be similar to other countries.

India

The P664L mutation was first identified in a FH homozygote of Asian Indian origin in the Gujerat province of India (Soutar et al. 1989). This mutation occurs at a CpG dinucleotide and creates a *Pst* I site in exon 14 of the LDLR gene. Two different haplotypes were found in association with P664L in FH patients in the UK, suggesting that the mutation arose independently in these cases (King-Underwood et al. 1991). This mutation, also identified in South African Indians by Rubinsztein et al. (1992), was probably spread across all over the world wherever the Muslim Gujeratis settled.

China

There are more than 50 different ethnic groups in China, of which the Hans forms the majority. The Hans Chinese originated in the Yangtze and the Hwang Ho valleys. Although China is a relative isolated population over many generations, no evidence for a founder gene effect has been found in the Jiang-su province where the majority of inhabitants are from the Yangtze valley group (Sun et al. 1994). An interesting observation is the apparently low plasma cholesterol levels and lack of xanthomata or premature CHD in Chinese FH heterozygotes studied by Sun et al. (1994). Mak et al. (1998a), however, observed the same clinical features in Chinese patients in Hong Kong as reported in Western countries or Japan,

and have also described three possible common mutations, C308Y, L393R and V408M, in this patient group. The L393R mutation accounts for 7.1% of FH cases in the Hong Kong group and occurred on the same haplotype as described in German patients. Due to the geographical distance and historic ethnic differences, it is reasonable to assume that mutation L393R arose independently at the CpG hotspot (Mak et al. 1998b).

Japan

Five LDLR gene mutations were found to underlie the disease in 30% of Japanese FH patients: 1845+2T→C (13.3%), C317S (6.7%), K790X (6.7%), P664L (3.3%) and E119K (1.7%). Mutation 1845+2 T→C has so far been found only in the Japanese population. Mutation C317S appears to be localised to the Kansai district, although the other mutations were widely distributed all over Japan. Since Japanese people are almost uniracial, and Japan is geographically isolated (Maruyama et al. 1995), these mutations may be considered founder-type sequence changes.

1.4 Diagnosis of FH

FH may be suspected in individuals with high cholesterol concentrations, normal triglyceride levels, tendon xanthomas and a family history of CHD. Early diagnosis of FH permits timely treatment which may prevent of delay the development of CHD. Although the FH phenotype lends itself to clinical diagnosis, not all cases can be diagnosed with certainty due to clinical variability of the disease, particularly in FH heterozygotes (Thompson et al. 1989, Kotze et al. 1993a, Gudnason et al. 1994, Pimstone et al. 1998). Sometimes the clinical expression of a given mutation varies within a family (Hobbs et al. 1989, Kotze et al. 1993b). Genetic

and environmental factors which may confer additional atherosclerotic risk to FH heterozygotes, such as elevated Lp(a) levels, hypertension, high blood homocysteine levels, diabetes, elevated iron levels, diet, old age and smoking may influence the disease expression and make prediction of CHD risk generally difficult. A definite diagnosis of FH can be established by the application of genetic testing to define the disease-causing mutation. DNA-based tests involve the screening of gross gene rearrangements by Southern blot analysis and/or long-range PCR, while point mutations, small deletions and insertions are usually detected by single strand conformation polymorphism (SSCP) analysis (Orita et al. 1989), denaturing gradient gel electrophoresis (DGGE) (Sheffield et al. 1989), RNase A cleavage (Myers et al. 1985) and/or chemical cleavage analysis (Cotton et al. 1988). Known mutations that predominate in specific population groups can be identified by restriction enzyme digestion of PCR products (Saiki et al. 1985) where appropriate, or allele-specific PCR (Newton et al. 1989). Kotze et al. (1995) have developed a multiplex PCR to identify multiple LDLR gene mutations in a single test.

In this study, a combined heteroduplex-single strand conformation polymorphism (HEX-SSCP) method (Kotze et al. 1995) was applied to screen for unknown mutations in the promoter and coding region of the LDLR gene. Denaturing gradient gel electrophoresis (DGGE), performed according to Nissen et al. (1996), was applied in cases where no mutations could be identified using the HEX-SSCP method. Direct sequencing was subsequently performed to identify mutations in PCR products demonstrating mobility shifts on polyacrylamide gels. The detailed experimental procedures used in this study are described in the form of flow charts in Appendix 1.

Aims of Study

Evaluation of the types and frequencies of mutations in different ethnic groups may broaden knowledge on the disease patterns in different population groups. The present study focussed on extensive DNA analysis of patients diagnosed with FH, in order to:

- investigate the molecular basis and mechanisms underlying inherited familial hyperlipidaemias in different population groups
- identify disease-causing mutations in hypercholesterolaemic index patients and subsequently in their relatives as part of the MED-PED initiative
- compare the spectrum of LDLR gene mutations in different South African populations
- develop a cost effective population-directed molecular screening strategy for FH in the South African population
- contribute to the establishment of a comprehensive predictive diagnostic service for FH in South Africa

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Chapter 2

Novel stop mutation causing familial hypercholesterolemia in a Costa Rican family

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Abstract

Combined heteroduplex single-strand conformation polymorphism (HEX-SSCP) analysis of the promoter and coding region of the low density lipoprotein receptor (LDLR) gene revealed a novel C to T mutation at nucleotide position 2056 in a Costa Rican patient with heterozygous familial hypercholesterolemia (FH). This nonsense mutation, Q665X, results in a termination codon in the epidermal growth factor (EGF) precursor homology domain of the mature LDLR.

Introduction

Familial hypercholesterolemia (FH) is an autosomal dominant disease caused by mutations in the low density lipoprotein receptor (LDLR) gene.[1] FH is characterised by raised plasma LDL cholesterol levels, tendon xanthomas and premature coronary heart disease (CHD).[2] The prevalence of FH is about 1 in 500 in most populations. Characterisation of FH mutations is important for definite diagnosis, genetic counselling and optimal treatment of the disease in affected families.

Materials and Methods

Blood samples were obtained with informed consent from two members of a Costa Rican family. Both were diagnosed with heterozygous FH based on clinical features and elevated LDL cholesterol levels (>5 mmol/l).[2] Levels of total plasma cholesterol (TC), high density lipoprotein cholesterol (HDLC) and triglycerides (TG) were determined by standard techniques as previously described.[3]

Genomic DNA was amplified by the polymerase chain reaction (PCR), using 20 sets of oligonucleotide primers according to Jensen *et al.* (1996).[4] PCR amplified fragments were screened by combined heteroduplex single-strand conformation polymorphism (HEX-SSCP) analysis according to Kotze *et al.* (1995).[5] Direct DNA sequencing of PCR products was performed using Sequenase Version 2.0 T7 DNA polymerase (United States Biochemical) according to the manufacturer's instructions. Restriction enzyme analysis of exon 14 PCR products was performed by *Aci*I digestion, electrophoresis in a 10% polyacrylamide gel and ethidium bromide staining. The exon 14 PCR was performed using primers 14.3 (5'-CCTGACTCCGCTTCTTCTGCC-3') and 14.4 (5'-ACGCAGAAACAAGGCGTGTGC-3').[1]

Results and Discussion

Mutation analysis showed a SSCP in exon 14 of the LDLR gene. Direct DNA sequence analysis revealed a novel C to T mutation, designated Q665X. This nonsense mutation at nucleotide position 2056 was predicted to destroy an AciI restriction enzyme cutting site, and this was confirmed in the DNA of the index case and her affected brother. After *Aci*I digestion of the PCR product, the normal allele was cut into fragments of 113 bp, 83 bp and 8 bp, while an additional fragment of 196 bp was observed in the FH patient heterozygous for mutation Q665X (Fig. 1). This nonsense mutation, which occurred in the second functional domain of the LDLR, is predicted to affect protein production or function. A truncated protein that would be rapidly degraded in the extracellular space is likely to be synthesised.[1, 6] The mutation may also lead to skipping of the exon in which it is located.[7]

We have recently described an 8 bp insertion in a Costa Rican FH family, revealing an insertional hotspot in exon 4 of the LDLR gene.[3] Although knowledge on the molecular

basis of FH in Costa Rica is limited, the identification of yet another novel LDLR gene mutation in the family analysed suggests that this population has its own spectrum of FH-related mutations. The Q665X mutation was absent in 10 unrelated Costa Rican FH patients after *Aci*I digestion of PCR-amplified DNA.

Fig. 1. Identification of a novel mutation, Q665X, in exon 14 of the LDLR gene. *Aci*I digested PCR products of a normal control (lane 1) and the patient (lane 2).

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Chapter 3

Mutation analysis in a small cohort of New Zealand patients originating from the United Kingdom demonstrates genetic heterogeneity in familial hypercholesterolaemia

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Abstract

Familial hypercholesterolaemia (FH) and familial defective apolipoprotein B-100 (FDB) are relatively common lipid disorders caused by mutations in the low density lipoprotein receptor (LDLR) and apolipoprotein B100 (apo B) genes, respectively. Molecular analysis at these loci were performed in eight Caucasoid New Zealand subjects with clinical features of heterozygous FH. Utilisation of an *in vitro* lymphocyte receptor assay demonstrated normal receptor function in four patients, of whom three screened positive for the founder-type apo B mutation, R3500Q, causing FDB. Four patients with reduced LDLR function, consistent with heterozygous FH, revealed 3 previously documented mutations in exons 3 (W66X), 6 (C292Y), and 7 (G322S) of the LDLR gene and a novel 2-bp deletion (TC or CT) after nucleotide 1204 (or 1205) in exon 9. The remaining patient was found to be FH/FDB negative after extensive mutation screening using both denaturing gradient gel electrophoresis (DGGE) and heteroduplex-single strand conformation polymorphism (HEX-SSCP) analysis. Haplotype analysis at the LDLR and apo B loci finally excluded the likelihood that mutations in these two genes underlie the FH phenotype in the molecularly uncharacterised family. This study demonstrated both mutational and locus heterogeneity in a small cohort of New Zealand hypercholesterolaemics originating from the United Kingdom.

Introduction

Apolipoprotein (apo) B is the major protein component of low-density lipoprotein (LDL) and mediates binding and uptake of LDL by its receptor on the cell surface.[1] Defects in the apo B-100 gene may prevent binding of the LDL to the receptor[2,3] and result in a phenotype designated familial defective apolipoprotein B 100 (FDB), that is clinically indistinguishable from familial hypercholesterolaemia (FH).[4] Both FH and FDB are characterised by raised LDL cholesterol levels, the presence of tendon xanthomata and premature coronary atherosclerosis[2], and occur at an estimated prevalence of 1 in 500 individuals.

Accurate diagnosis of FH and FDB can be performed by screening genomic DNA for mutations in the LDLR- and apo B genes. Although FDB is usually caused by mutation R3500Q[5] or a less common mutation R3531C,[6] undetermined mutations in the apo B gene may explain some presentations of autosomal dominant hypercholesterolaemia (ADH). ApoB - LDLR interactions may also be affected by processes such as antibody binding[7] to either the LDLR or apo B moiety. The potential heterogeneity of abnormalities of receptor-ligand binding suggests that the clinical syndrome characterised by very high LDL cholesterol levels could be diverse for molecular aetiology. A more precise determination of the molecular basis of phenotypic familial hyperlipidaemia can be addressed through specific assays measuring LDLR activity and by LDL binding assays. The finding of normal receptor activity in a patient with very high cholesterol levels indicates a non-receptor defect, and further molecular studies are then warranted to exclude FDB or other LDL binding abnormalities. In this study 8 New Zealand patients from Anglo-Saxon origin were screened for mutations in the LDLR and apo B genes, in order to define the spectrum of mutations underlying the clinical phenotype of heterozygous FH in these subjects.

Materials and Methods

Patients

A total of 8 unrelated hypercholesterolemic individuals, attending the Lipid Disorders Clinic at the Christchurch Hospital in New Zealand, were the subjects of the study. These Caucasoid New Zealanders of Anglo-Saxon origin were diagnosed with heterozygous FH, based on the presence of tendon xanthomata and a parental history of hypercholesterolaemia. All patients had normal renal, hepatic and thyroid function.

Biochemical Analysis

Blood samples were collected for measurement of lipids after a 12 hour fasting period, following 6 weeks of an extensive diet. Hypolipidemic drug therapy was withdrawn 6 weeks prior to blood sampling. Plasma was used for lipid and apolipoprotein determinations, whereas blood cells were utilised for DNA preparation[8] in the Hunter-Nye lipid Laboratory in Dunedin (NZ). Total plasma cholesterol (TC) and total triglycrides (TG) were measured using enzymatic kits (Boehringer Mannheim) and controls from the Australian Lipid Standardisation programme. High density lipoprotein cholesterol (HDLC) was measured in the supernatant obtained after precipitation of lipoproteins containing apolipoprotein B from plasma. LDL cholesterol levels were calculated using the Friedwald equation.[9]

LDLR activity

Specific assays measuring LDLR activity and LDL binding assays were assessed using the method of Cuthbert *et al.*[9] A complete description has been given elsewhere.[10]

Mutation detection

Four patients who exhibited normal LDLR function were screened for FDB as previously described.[11] Patients without mutations in the apo B gene were subjected to extensive mutation screening of the LDLR gene. Polymerase Chain Reaction (PCR)[12] amplification of the promoter and coding regions were performed, using 20 sets of exon-specific primers according to Jensen *et al.*[13] The study population has previously been screened for mutations in exon 4 of the LDLR gene[10] and therefore this part of the gene was excluded from the analysis. PCR-amplified fragments were screened by combined heteroduplex single-strand conformation polymorphism (HEX-SSCP) analysis according to Kotze *et al.*[14] which was performed on three different polyacrylamide gel systems in order to increase mutation detection efficiency. Direct sequencing of PCR products that revealed aberrant electrophoresis patterns were performed by the dideoxy-chain termination DNA sequencing method using a commercial kit (Sequenase version 2.0 DNA sequencing kit) and/or fluorescence cycle sequencing followed by detection on a Perkin Elmer ABI PRISM 310 sequencer. Detection of mutations W66X, C292Y and G322S were confirmed by *Xba*I, *Rsa*I and *Bgl*I restriction enzyme analysis, respectively. Restriction-enzyme digested PCR products were resolved on a 10% polyacrylamide gel (3%C) and visualised by staining with ethidium bromide.

Haplotype analysis

Haplotype analysis using polymorphic markers at the apo B [variable number of tandem repeats (VNTR), dinucleotide TG $(TG)_n$, signal peptide (SP) markers][15-17] and LDLR [D19S394, D19S583, dinucleotide TA $(TA)_n$][18-20] loci was performed in the family of patient KD. No mutations could be identified in this patient after extensive DNA screening using both HEX-SSCP analysis and DGGE performed according to Nissen *et al.*[21] Blood samples were obtained with informed consent from seven family members for the haplotype analysis.

Results and Discussion

The demographic and clinical characteristics of 8 hypercholesterolaemic subjects are summarised in Table 1, together with disease-related mutations identified in the apo B and LDLR genes. LDLR studies demonstrated reduced receptor mediated uptake of LDL consistent with heterozygous FH in subjects IB, ST, JS and JB, in whom LDLR gene mutations were identified, while the remaining patients had completely normal receptor function on repeated assay (data not shown). Detection of the common apo B mutation R3500Q causing FDB in subjects AW, RA and PR was compatible with these results. TC levels ranged from 9.4 to 10.3 mmol/l in these three patients, and from 7.8 mmol/l to 9.9 mmol/l in the four FH patients. It is noteworthy that the TC levels are generally higher in the FDB patients than in the FH patients, since previous studies suggested that FDB may be associated with a milder disease phenotype.[22, 23]

The four different mutations identified in the LDLR gene are illustrated in Figure 1. The three previously-described point mutations in exons 3 (W66X),[24] 6 (C292Y)[24] and 7 (G322S)[3] alter known restriction enzyme recognition sites and could be confirmed in genomic DNA using XbaI, RsaI and BglI, respectively. The novel 2-bp deletion (TC or CT) identified after nucleotide 1204 (or 1205) creates a stop codon at residue 418. This deletion most likely causes the FH phenotype in patient IB, since the translational frameshift would result in a truncated protein lacking essential domains of the receptor. Family members were not available to confirm Mendelian inheritance of the LDLR gene mutations identified in this study, but these mutations were absent in 100 normal chromosomes screened by HEX-SSCP analysis.

The remaining patient with normal LDLR activity, but without a known FDB mutation, presented with a total cholesterol level of 7.8 mmol/l after following a cholesterol-lowering diet for 6 weeks. Haplotype analysis in the family of this patient (KD), using highly polymorphic markers at the LDLR and apo B genes, finally reduced the likelihood that mutations in these

genes are responsible for the disease phenotype. Although limited data are available in this relatively small family, it is clear that the two normocholesterolaemic children of the index patient inherited different chromosomes from their father (Figure 2).

Exclusion of FH and FDB in family KD after extensive mutation screening and haplotype analysis, is in accordance with earlier observations that there remains a proportion of clinical FH patients in whom no detectable defects can be identified in the LDLR or apo B genes,[25-27] suggesting that there might be a third gene underlying the FH phenotype. The combined use of HEX-SSCP and DGGE mutation screening is expected to detect up to 100% of small mutations. This family was therefore included in the linkage analysis performed by Varret et al. (1999),[28] using microsatellite markers D1S2892 and D1S2722, shown to map near a third locus implicated in ADH. The likelihood that the putative gene on chromosome 1 causes the FH phenotype in family KD was, however, also excluded.[28] Studies are underway to determine whether the hypercholesterolaemia in this family is caused by yet another locus recently implicated in ADH (M. Varret et al., unpublished results).

The demonstration of both mutational and locus heterogeneity in ADH in the small cohort of New Zealand hypercholesterolaemics studied complicates DNA diagnosis of FH in this population. However, the fact that the founder-type apo B gene mutation, R3500Q, was detected in three of the molecularly characterised hypercholesterolaemics studied, implies that a significant proportion of affected Caucasians living in New Zealand may have FDB. Although screening of a larger population sample needs to be undertaken to confirm this, future molecular diagnostic approaches in this population should first focus on detection or exclusion of mutation R3500Q. The apparently high prevalence of FDB in New Zealand hypercholesterolaemics originating from the United Kingdom may be due to a founder effect, similar to the situation

described for FH in South Africa, where three LDLR gene mutations (D154N, D206E and V408M) are responsible for the disease in 90% of Afrikaners of European descent.[29] FDB was found to be rare in South Africa, most likely as a consequence of a "negative" founder effect which diluted the frequency of mutation R3500Q in the settlers relative to their parent European populations.[30] Notably, the most common mutation D206E detected in South Africa, causing FH in approximately 60% of Afrikaners, has been identified in only one of the New Zealand FH patients screened for mutations in exon 4 of the LDLR gene.[10]

This study highlighted the value of DNA-based diagnostic methods in the complete elucidation of the genetics of ADH. The identification of new genes causing the FH phenotype may lead to the development of novel therapeutic approaches to prevent cardiovascular disease, which remains the leading cause of death in many countries.

Table 1 Demographic and clinical data of the probands screened for mutations in the apo B and LDLR genes

Subjects	Sex	Age (yrs)	XMTA	CHD	TC (mmol/l)	TG	HDLC	LDLC	Mutation	Reference
Normal LDLR function: Apo B gene										
AW	F	42	+	+	9.4	0.6	1.4	7.7	R3500Q	5
RA	M	48	+	+	10.3	2.7	1.2	7.9	R3500Q	5
PR	F	49	+	-	10.2	1.1	0.9	8.8	R3500Q	5
KD	M	59	+	+	7.8	2.5	1.3	5.3	None	
Reduced LDLR function: LDLR gene										
IB	M	31	+	-	8.9	1.4	1.3	6.9	1204del2	New
ST	M	41	+	-	9.9	1.8	1.2	7.9	C292Y	24
JS	M	43	+	-	9.8	1.1	1.2	8.1	W66X	24
JB	F	69	+	+	9.8	1.0	1.8	7.4	G322S	3

XMTA, tendon xanthomas; CHD, coronary heart disease

Figure 1 Identification of mutations in the LDLR gene. **A**, Automated fluorescent sequence analysis demonstrates a 2-bp TC or CT deletion in FH affected individual IB. **B**, Restriction enzyme analysis of PCR products of exons 3 (XbaI), 6 (RsaI) and 7 (BglI). FH affected individuals are indicated in lanes 1, 3 and 5 for the respective exons. PCR products of normal controls was loaded in lane 2 (exon 3), lane 4 (exon 6) and lane 6 (exon 7).

Figure 2 Segregation analysis of the LDLR and Apo B loci in family KD (II:3). Pretreatment total cholesterol (TC), triglycerides (TG), high density lipoprotein (HDL) and low density lipoprotein (LDL) levels in mmol/l together with the respective ages (in years) of family members are indicated below the pedigree symbols. The disease status of III:3 was uncertain. MI, myocardial infarction; nd, not determined.

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Chapter 4

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 due to 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, 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 frequency ($P<0.05$) in FH patients compared to controls, and occurred *in cis* with mutation E387K in one family. Analysis of four intragenic LDLR gene polymorphisms demonstrated 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.

Introduction

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 (apo B) 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 premature coronary heart disease (CHD). The estimated incidence of both FH and FDB is approximately 1 in 500 in most Caucasian 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 Nguni's (Xhosa, Tembu, Swazi and Zulu) along the east coast, the Sotho's (South Sotho, North Sotho/Pedi, West Sotho/Tswana) who settled further west on the Transvaal highveld, and the Venda's living in the Northern Transvaal area.[6, 7] We suspect that FH is not frequently recognised in Africans due to 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 germ-line mutations is 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 due to a "negative" founder effect that diluted the frequency of the common apo B3500 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 by 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 apo B gene. Blood samples were also obtained from 38 of their family 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 and/or premature CHD in the index case or a first-degree relative. Probable FH (4 probands) was defined by the same pretreatment cholesterol level and primary hypercholesterolaemia and/or premature CHD 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 individuals drawn from the same population (19 Pedi's, 21 Sotho's, 27 Xhosa's, 29 Zulu's) were sampled as controls. TC, high-density lipoprotein cholesterol (HDLC) and triglyceride

(TG) determinations and extraction of genomic DNA were performed using standard methods[13] Plasma LDL-cholesterol (LDLC) concentrations were calculated with the Friedewald formula [LDLC=TC-(HDL+TG/2.18)].[14]

Mutation detection

Heteroduplex single-strand conformation polymorphism (HEX-SSCP) analysis was performed in South Africa,[15] and denaturing gradient gel electrophoresis (DGGE) in Denmark[16] and Scotland,[17] to screen polymerase chain reaction (PCR)-amplified genomic DNA for mutations in the LDLR and apo B 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'-CCACGTCATTTACAGCATTTCAATG -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) and/or an automated sequencer ABI 373A.

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, revealed 6 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+/StuI+/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 E387K[19] 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 apo B 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 apo B gene have previously been reported in 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 apo B mutation at codon 3540. RFLP analysis indicated that haplotype SmaI+/StuI+/AvaII-/NcoI+ co-segregated 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 apo B 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 demonstrated 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. Individual 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, revealed 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 control individuals from the general Black population comprising 56 Nguni's (27 Xhosa's, 29 Zulu's) and 40 Sotho's (19 Pedi's, 21 Sotho's) resulted in the identification of 6 individuals [4 Nguni's (1 Xhosa, 3 Zulu's) and 2 Sotho's (1 Pedi, 1 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 Nguni's and 2/10 Sotho's with FH) compared to the controls (4/56 Nguni's and 2/40 Sotho's). 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 ($\chi^2=5.916$, 1df, $P=0.0149$). We furthermore identified 5 carriers of the -175g→t polymorphism among 40

lipid clinic patients without the FH phenotype (13%), demonstrating an intermediate allele frequency of 0.06. This was not significantly different from the frequencies observed in the FH (χ^2=1.326, 1df, P=0.249) or control (χ^2=1.474, 1df, P=0.224) groups. Variant -175g→t was also detected in 1/47 DNA samples of control individuals from the Venda tribe studied by Ehrenborg et al.[25] while absent in more than 300 Caucasians screened.[26]

Discussion

Numerous LDLR gene mutations (>600) have been identified in FH patients world-wide, 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 [19, 23, 28, this study] 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 the LDLR, and impairs its transport but not lipoprotein binding in fibroblasts.[23] Frequent detection of a deleterious mutation can be due to 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 Tswana's) on the same haplotype, have not (yet) been reported in other populations. These findings largely exclude the likelihood of a recurrent mutational event due to 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 prior to

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 and colleagues[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 heterozygous 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 endotoxemia and severe gram-negative infections[32] 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 demonstrate that the -175g→t polymorphism residing in a *cis*-acting element in the LDLR promoter[34] does not cause the FH phenotype in affected individuals, further studies are warranted to investigate the likelihood that this variant may influence disease expression. The likelihood that the significantly higher frequency of the -175g→t promoter polymorphism in South African Black FH patients compared with controls ($P<0.05$) is caused by linkage disequilibrium with another downstream mutation causing the FH phenotype, was excluded by haplotype studies which demonstrated that the rare t allele was associated with different LDLR haplotypes. This allele furthermore co-segregated with missense mutation E387K in one family. These different

chromosomal backgrounds may be the result of recombination events, reflecting the age of the -175g→t variant. Compared to Caucasians, Blacks are considered older in evolutionary terms[35] and can therefore be expected to have accumulated variation over a longer time period. It is possible that the -175g→t polymorphism did not spread to other parts of the world, hence its apparent absence in Caucasian populations.[18, 36, this study] The African origin of the -175g→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 due to 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 demonstrated 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 and/or environmental factors.

Although the identified missense mutations have not yet 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 due to 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 condition[1] 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. Elevated 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.[42, this study] None of the FH heterozygotes with the relatively severe 6-bp deletion in exon 2[23] 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 due to interaction with other genetic and environmental factors, including a prudent diet.[11] Data provided by us and others[43-45] 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.

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

Index	Ancestry	Sex	Age	TC	TG	HDL	LDL	Clinical	LDLR gene sequence changes	Apo B gene sequence changes	Relatives Tested
				(mmol/l)[c]							
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[a]	Swazi/Zulu	F	56	8.3	1.5	1.9	5.7	Arc			11
CK[b]	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[b]	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[a]	Pedi	F	57	7.4	2.6	0.9	5.3	Arc, PVD			6
RM[a]	Pedi/Tswana	F	54	10.8	0.4	1.3	9.3	Arc, ?Xan			3
LM[a]	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.
[a]Probable FH; [b]Clinical FH homozygotes; [c]Pretreatment concentrations, except for proband LM for whom pretreatment levels was not available

Figure 1 Pedigree of proband EF (arrow) clinically diagnosed with heterozygous FH. Clinical, biochemical and genetic data are provided for individuals of whom DNA samples were available. Those with elevated 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.

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Chapter 5

The genetics of familial hypercholesterolaemia in South Africa: Multiple founder mutations underlie the high disease prevalence in a diverse population

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In Preparation

Abstract

Familial hypercholesterolaemia (FH) has been a focus of research in South Africa since 1980, when this lipid disorder was recognised as a common cause of premature coronary heart disease (CHD) in Afrikaners of European descent. Extension of clinical and biochemical studies to also include molecular analysis of the low-density lipoprotein receptor (LDLR) gene, revealed 65 different mutations in South African hypercholesterolaemics. In addition to mutations D154N, D206E and V408M which account for FH in 90% of affected Afrikaners, four missense mutations D200G, S285L, C356Y and G361V representing possible minor founder events, were identified in this relatively homogeneous population group. Common mutations have also been described in South African Indians (P664L) and Jews (del 197), most likely as a consequence of multiple introductions of defective genes into these isolated communities, following earlier expansion in the parent countries. Subsequent mutation analysis of the indigenous South African population of mixed ancestry revealed that the aforementioned LDLR gene mutations contribute significantly (~20%) to their FH phenotype. Predominance of a 6-bp deletion identified in exon 2 of the LDLR gene in the Black population, where FH appears to be extremely rare, raises the possibility that heterozygous FH may be associated with a selective advantage in Africa. Delineation of the mutational spectrum in South African FH patients has revealed that variation in the LDLR promoter region may significantly influence the expression of FH-related gene defects in indigenous African populations where these mutations prevail.

Introduction

Familial hypercholesterolaemia (FH) contributes significantly to the high mortality rate from coronary heart disease (CHD) worldwide (Goldstein et al. 1995). To date, more than 600 mutations (http://www.ucl.ac.uk/fh and http://www.umd.necker.fr.) underlying this common lipid disorder have been identified (~1/500) in the low-density lipoprotein receptor (LDLR) gene. Despite the general diversity of mutations responsible for FH, some mutant alleles are common in specific South African ethnic groups (Kotze et al. 1991a, 1998, Meiner et al. 1991), which serve as a helpful tool in the implementation of population-directed genetic screening programs. Founder effects with or without genetic drift probably resulted in an increased prevalence of FH in Afrikaners (Seftel et al. 1980) and several other homogeneous populations groups (Lehrman et al. 1987, Moorjani et al. 1989), but are unlikely to occur in countries with great ethnic and genetic diversity. FH is not associated with infertility, and therefore the gene frequency would not be influenced by negative selection (heterozygote disadvantage) in the heterozygous state.

This review will focus on new perspectives derived from delineation of the mutational spectrum of the LDLR gene in the South African population, known to be enriched with specific founder LDLR gene mutations. Although these common mutations impose a major risk for the development of CHD, several advantages could be derived for diagnostic and research purposes. The unique South African Afrikaner community is a valuable candidate population for mapping of genetic traits, analysis of geographic distribution and origins of mutations, investigation of new causes of the FH phenotype in patients without founder-related mutations, delineation of gene-gene interactions, evaluation of factors modulating phenotypic expression of the disease, and studies of genetic determinants of therapeutic response (Davigon and Roy, 1993).

Exploiting the "founder effect" in the Afrikaner population

The Afrikaner population of South Africa provides an excellent example of founder events in human evolution. The high prevalence of several genetic diseases in this population (http://helix.biology.mcmaster.ca/3j3/3j3.founder) resulted from a restriction in genetic variability due to geographic isolation and strict religious/cultural bonds, coupled to rapid population expansion over 10-12 generations. Molecular-genetic research of FH in South Africa was initiated in 1986, in an attempt to investigate the founder hypothesis (Seftel et al. 1980) implying that a limited number of LDLR gene mutations would underlie the disease in the Afrikaner population. Initially, intragenic restriction fragment length polymorphisms (RFLPs) were used to define haplotype associations, demonstrating a predominance of three distinct chromosomes in the FH population compared with a control group (Kotze et al. 1987, 1989a, Leitersdorf et al. 1989). Subsequent cloning and/or DNA sequence analysis of the disease-associated chromosomes led to the identification of three founder-related point mutations, D154N, D206E and V408M, collectively accounting for the disease in ~90% of affected Afrikaners (Kotze et al. 1989b, 1990, 1991a, Leitersdorf et al. 1989). Based on this knowledge, a cost-effective polymerase chain reaction (PCR)-based method was developed for the simultaneous analysis of all three founder mutations in a single multiplex assay (Kotze et al. 1995). Direct mutation screening demonstrated that DNA diagnosis of FH is more accurate than a clinical diagnosis (Kotze et al. 1992, Koivisto et al. 1992). Genotype-phenotype correlation studies performed in the Afrikaner population furthermore demonstrated for the first time that mutational heterogeneity in the LDLR gene may influence the phenotypic expression of heterozygous FH (Kotze et al. 1993a, Graadt van Roggen et al. 1995). However, other genetic and environmental factors were found to contribute to

expression of disease-causing mutations (Kotze et al. 1993b, Pimstone et al. 1998), which complicates accurate diagnosis of FH.

To assure continuing awareness of this treatable disorder in South Africa and to highlight the importance of family studies, particularly in cases where the gene defect has been identified in an index patient, two Genetic Information Meetings were held during 1998. These events were an extension (Kotze et al. 1996a) of the international MED-PED project (Williams et al. 1993). This initiative also served as a paradigm of other common genetic diseases in South Africa (Kotze and Callis 1999).

Population screening to define the mutational spectrum in South Africa

Since 1994, the focus of FH research in South Africa has been aimed at the development of a comprehensive population-directed molecular test for accurate diagnosis of this disease. Knowledge of the mutational spectrum underlying FH in different South African ethnic groups is a prerequisite for such a screening approach. Therefore, DNA samples of FH patients without any of the previously-described founder-type mutations (Kotze et al. 1991a, 1998, Meiner et al. 1991, Kotze et al. 1997, Loubser et al. 1999), were subjected to extensive mutation analysis of the promoter and coding region of the LDLR gene. A total of 65 different LDLR gene mutations, including ten founder-type mutations highlighted in Table 1, have been identified in the diverse South African population. Predominance of a limited number of LDLR gene mutations in specific groups facilitated the development of a cost-effective population-directed screening strategy for accurate diagnosis of FH in South Africa.

Afrikaner population: Identification of four additional "minor founder" LDLR gene mutations

Previous mutation and haplotype studies demonstrated that the three common Afrikaner mutations, identified in exons 4 (D154N, D206E) and 9 (V408M) of the LDLR gene, were probably introduced into South Africa from the United Kingdom and the Netherlands, respectively (Gudnason et al. 1993, Defesche et al. 1993). Extended mutation analysis of Afrikaner FH patients without these three common mutations revealed 10 additional sequence changes in this population (Table 1). Four missense mutations, D200G, S285L, C356Y and G361V, occurred in 12 apparently unrelated Afrikaner families. Notably, in a study performed approximately ten years ago four unique FH-associated haplotypes were identified (Kotze et al. 1989a), which defined the chromosomal backgrounds for each of these "minor founder mutations". In order to further assess the likelihood of a common origin or recurrent mutational events in all the above cases, haplotype studies were extended using two highly informative microsatellite markers, D19S221 and D19S394, located 1.3 Mb centromeric and 250 kb telomeric to the LDLR gene, respectively (Traeger-Synodinos et al. 1998). In general, the genotype results were consistent with a common ancestry for each mutation. Minor differences in allelic sizes detected in a small proportion of the samples, can probably be attributed to replication slippage within the repeats. Unequal crossing-over events are highly unlikely in this context, because the differences in allele sizes involved only single repeats. Similar results were obtained for 13 patients with mutation D154N, 25 with D206E and 6 with V408M, and therefore it was not possible to make any predictions on the historical ages of these sequence changes. However, mutation S285L in exon 6 was probably introduced/arose most recently in the Afrikaner population, since association with identical

alleles for both D19S221 and D19S394 was demonstrated in Afrikaner families with this mutation.

Previous detection of mutations D200G and S285L in the United Kingdom and the Netherlands respectively, indicate that their mutational origins can probably be traced back to these countries (Hobbs et al. 1992, Gudnason et al. 1993), although both the mutations have also been observed in other populations (http://www.ucl.ac.uk/fh). In contrast, novel mutations C356Y and G361V have not previously been described in other populations. None of the mutations were identified in the normal population, and both involve evolutionary conserved residues (Mehta et al. 1991) in the epidermal growth factor (EGF) precursor homology domain of the LDLR gene. This domain mediates acid-dependent dissociation of the receptor and ligand in the endosome and therefore mutations in this LDLR region are likely to affect receptor recycling (Hobbs et al. 1992). Mutations D200G, S285L, C356Y and G361V co-segregated with hypercholesterolaemia in the respective families. All the other mutations detected in single Afrikaner families have been described previously, and were confirmed by restriction enzyme analysis where appropriate. Identification of at least seven founder-related LDLR gene mutations among Afrikaners may imply that the reported FH prevalence of 1/70 in Afrikaners, based on screening for three mutations causing FH in 90% of cases and a background prevalence of 1/500 (Steyn et al. 1997), may be an underestimate.

Mixed ancestry: Caucasoid admixture contributes significantly to the FH phenotype

The prevalence of FH has not yet been determined in South Africans of mixed ancestry, but the clinical impression is that it is relatively high. Loubser et al. (1999) have demonstrated that three Afrikaner founder mutations contribute significantly (15%) to the FH phenotype in this indigenous South African ethnic group (Coloured population). Mutations D154N and

D206E were associated with the same haplotype in the Coloured and Afrikaner populations, indicating a common origin. Detection of mutation V408M on different chromosomal backgrounds can probably be ascribed to a recombination or gene conversion event (Loubser et al. 1999). The 3-bp deletion of codon 197 found to be common in Aszkenazi Jews and mutation P664L that is frequently detected in Indians (Table 1), were probably introduced into the Coloured population by recent admixture. These mutations, together with the Afrikaner founder mutations and a 2.5-kb deletion involving exons 7-8 (4%), account for FH in at least 20% of affected Coloureds living in the Western Cape Peninsula (Loubser et al. 1999). In one of the probands of mixed ancestry, a promoter mutation (-59c→t) was identified within the 10-bp core sequence of repeat 2, which resulted in reduced LDLR transcriptional activity (Scholtz et al. 1999). This mutation was detected in her normocholesterolaemic son, together with a single base change at nucleotide position –124 (c→t) which increases LDLR promoter activity (~160%). The intra-familial phenotypic variability observed amongst individuals with the -59c→t mutation has therefore been ascribed to allelic interaction, suggesting that variation in the LDLR promoter region may significantly influence the expression of FH-related mutations in populations where these mutations prevail. Notably, polymorphic sequence variants are frequently detected in the LDLR promoter region in populations with an African genetic element, but appear to be absent in Caucasians (Top et al. 1992, Hoogendijk 1999). The Coloured population appears to be an excellent example of the consequence of admixture in populations with different disease risks due to genetic factors, and is a potential candidate for admixture linkage disequilibrium (ALD) approaches to map genes involved in CHD and other complex traits.

Black population: Predominance of a 6-bp deletion

Hypercholesterolaemia in the South African Black population (Nguni's, Sotho's and Venda's) appears to be an unusual finding (Seedat et al. 1993). However, it has been reported that the prevalence of ischaemic heart disease is increasing among urban Blacks in South Africa, which can probably be ascribed to adoption of a western lifestyle. Interestingly, a 6-bp deletion (GCGATG) has been detected in exon 2 of the LDLR gene (Leitersdorf et al. 1988) in four apparently unrelated Tswana and Pedi patients. Two of these patients from different tribal groups were homoallelic FH homozygotes (Leitersdorf et al. 1988, Thiart et al. in press). The observation that this deletion is associated with the same haplotype in different tribes, indicates that this may be an ancient mutation predating tribal separation approximately 3000 years ago (Thiart et al. submitted). A promoter polymorphism at nucleotide position -175 (g→t) was detected at a significantly higher frequency in FH patients compared to controls drawn from the same population ($P<0.05$), suggesting that this variant might exacerbate the expression of other FH-related mutations in South African Black patients (Thiart et al. submitted). Future studies should indicate whether this –175 g→t variant detected exclusively in populations with an African genetic element (Hoogendijk 1999) might impose a health threat with westernisation and/or may be due to other selective forces involved in human evolution. Although plasma cholesterol levels were similar in subjects from the general population with and without this sequence variant, preliminary data has revealed a significant association between the -175t allele and diastolic blood pressure ($P=0.019$) (MJ Kotze and C Lombard, unpublished data).

South African Indian population: CpG hotspot mutations are a frequent cause of FH

The increased prevalence of FH in the South African Indian community (~1/100) has been attributed to a combination of recurrent mutational events, multiple entries of disease genes and/or a reflection of a founder gene mutation in India. Mutation P664L, originating from the Gujerat Province in the West of India, was found to be the most common mutation (47%) in South African Indians (Rubinsztein et al. 1992, Kotze et al. 1997). Further mutation analysis indicated that CpG hotspot mutations, such as P664L, are a frequent cause of FH in the South African Indian population. All the Indian FH patients with mutation P664L shared the same haplotype. Haplotype analysis of mutation E207K, detected in two apparently unrelated Indian families, suggested a common origin in India (Kotze et al. 1997). This mutation has previously been identified on different chromosomal haplotypes in genetically distinct populations (Leitersdorf et al. 1990, Hobbs et al. 1992).

South African Jewish population: High prevalence of FH due to the Lithuanian mutation

The South African Jewish population appears to have a particularly high incidence of FH, most likely as a consequence of multiple entries of defective genes into this relatively isolated community over recent years. An in-frame 3-bp deletion (Gly197), designated the FH Lithuania mutation, was reported to occur in the majority (80%) of South African Jews with FH (Meiner et al. 1991). However, in an extended analysis of Jewish FH patients living in South Africa, the Lithuanian mutation was identified in less than 50% of patients diagnosed with heterozygous FH (M Callis et al. unpublished data). This mutation has also been identified in many Ashkenazi Jewish families throughout the world (Meiner et al. 1991). Additionally, three novel mutations were identified in South African Jews, which include a

nonsense mutation Q104X, a missense mutation N407K and a splice-site mutation 1358+1G→A. Mutation Q104X results in a truncated protein causing the disease phenotype. Mutation N407K was identified in a patient who was also heterozygous for the common Afrikaner mutation D206E. The two daughters of this patient who inherited mutation N407K, presented with normal cholesterol levels (M Callis and FJ Raal, unpublished data). Mutation 1358+1G→A affects the invariant GT dinucleotide at the splice donor site and can therefore be expected to cause FH in the patient with this mutation.

European ancestry: identification of various private mutations

Eighteen private mutations were identified in individuals from different ancestries living in South Africa. These mutations may have been introduced into South Africa by recent immigrants or occurred as random events. These patients originated mostly from European countries, where admixture is not uncommon. Mutation 2140+5G→A (Heath et al. 1999) was identified in a patient of Irish descent, as well as a South African Jewish patient. Although this splice mutation does not involve a conserved base, it is associated with a consensus value of 0.84 (Krawczak et al. 1992). A novel complex mutation involving both a deletion and an insertion in the same gene region was detected in exon 10 in a Welsh patient. This mutation deletes sixteen bases from nucleotide 1379 to 1394 (ACGGCGTCTCTTCCTA), which is replaced by five bases (CAGCT). Six amino acids, His^{439}-Gly^{440}-Val^{441}-Ser^{442}-Ser^{443}-Tyr^{444}, were replaced by two amino acids, Pro^{439}-Ala^{440}. Interestingly, analysis of the sequence close to the mutation revealed that the CAGCT motif, inserted in the mutant LDLR gene, is found 11 nucleotides upstream of this mutation. The insertion results in a stop codon at amino acid position 441 and is therefore highly likely to affect LDLR function and cause FH.

Although founder events are highly unlikely in heterogeneous populations, some mutations may occur at an increased prevalence in certain geographical regions. These include the combined defect N543H/2393 del 9-bp identified in one of our probands originating from Northwest Netherlands, where this mutation is predominantly found (Defesche and Kastelein 1998), and those described in the United Kingdom (Day et al. 1997a) and Belgium (Descamps et al. 1997). The high frequency of these mutations not associated with an increased disease prevalence in the respective countries, may be attributible to geographical isolation.

Comparative analysis in different populations: Ratio of missense to stop mutations

The 65 mutations identified in the South African population occurred throughout the promoter and coding region of the LDLR gene (Fig. 1). These included 42 (65%) missense mutations, 6 (9%) nonsense mutations, 9 (14%) deletions, 2 (3%) insertions, 1 (2%) deletion/insertion, 3 (5%) donor splice-site mutations and 2 (3%) acceptor splice-site mutations. Most of the mutations occur in exons 4, 8 and 9. Exon 4 is known to be a "hotspot" area for LDLR gene mutations (Gudnason et al. 1994, Kotze et al. 1996b, Day et al. 1997b). Although deletions and insertions are frequently identified, combined deletional and insertional (ID) events are very rare. Such complex mutations have previously been reported in the LDLR gene (Yamakawa-Kobayashi et al. 1994, Koivisto and Kontula 1996). Mutations that occur in introns may affect splicing and make them an important target for molecular analysis (Peeters et al. 1999). Variation in the LDLR promoter region that may affect transcription, has so far only been detected in South Africans with an African genetic element.

The majority of sequence changes detected in the South African population are point mutations involving amino acid substitutions. Notably, Day et al. (1997b) have recently

reported a four-fold higher than expected ratio (1/5 vs 1/20) of stop mutations than missense mutations in FH patients. In the South African population the ratio of single base substitutions resulting in stop codons compared to those causing amino acids substitutions was 1/10. This may suggest that the local environment may readily "unmask" phenotypically less severe (compared to stop mutations leading to truncated proteins) LDLR mutations. This finding may be related to dietary habits of the local population (e.g. fatty diet in Afrikaners) and/or interaction with other sequence changes at the LDLR gene locus (e.g. promoter variants in FH patients with an African genetic element), or may be an indication that FH patients had an advantage during earlier times when infectious diseases were rampant in Africa. Support for the latter hypothesis came from the finding that LDLR-deficient mice are protected against certain infections (Netea et al. 1996), and reports that LDL may bind certain viruses and inhibit their infectivity (Feingold and Grunfeld 1997). Notably, Hobbs et al. (1990) were the first to speculate on the likelihood of a Darwinian selection that may favour the heterozygous FH state in Africa.

Familial defective apolipoprotein B-100 (FDB) in South Africa

Another common lipid disorder that is phenotypically indistinguishable from FH, familial defective apolipoprotein B100 (FDB), was found to be rare in South Africa (Kotze et al. 1991b, Rubinsztein et al. 1995). FDB is caused by mutation R3500Q, or less common mutations such as R3531C and R3500W in the apolipoprotein B100 (apoB) gene (Soria et al. 1989, Pullinger et al. 1995, Gaffney et al. 1995). Rubinsztein et al. (1993b) reported compound heterozygosity for FH mutation D206E and the FDB mutation R3500Q in a large Afrikaner pedigree. One family member had a combination of mutations D206E, V408M and R3500Q. Individuals with both FH and FDB presented with lipid levels and clinical features that are intermediate in severity between the heterozygous and homozygous FH state.

The R3500Q mutation was recently identified in a single FH patient of mixed ancestry (Loubser et al. 1999) and in an Afrikaner hypercholesterolaemic referred for molecular diagnosis of FH (E. Langenhoven, personal communication). Two silent mutations, T3540T and T3552T, previously described in the apo B gene in Nigerian and African American subjects (Pullinger et al. 1999) were identified in two South African Black individuals (Thiart et al. submitted).

Autosomal Dominant Hypercholesterolaemia (ADH)

Failure to identify FH- or FDB-related mutations in approximately half of the South African patients with a clinical diagnosis of heterozygous FH, despite the use of three sensitive mutation detection methods (Kotze et al. 1995, Nissen et al. 1996), can be considered a reflection of locus heterogeneity in ADH, as recently demonstrated by Varret et al. (1999) and Hunt et al. (1999). A new locus designated FH3 was localised to human chromosome 1p34.1-p32 in a number of families. Lack of linkage to the known ADH loci provided evidence that a fourth locus could underlie the FH phenotype. Families from all over the world were analysed in this study, including three families initially characterised at the DNA level in South Africa. This analysis included extensive mutation and haplotype analysis at both the LDLR and apo B gene loci (Thiart et al. submitted). The genetic heterogeneity of ADH was confirmed by several other studies (Haddad et al. 1999, Pajukante et al. 1998, 1999), and evidence was also provided for the existence of autosomal recessive hypercholesterolaemia (ARH) (Varret et al. 1999, Zuliani et al. 1999). These proposed novel genes probably underlie only a small proportion of primary hypercholesterolaemia in Afrikaners without the documented founder-type mutations, since LDLR gene mutations were detected in ~80% index cases subjected to extensive mutation analysis.

Conclusion

The systematic mutation screening strategy conducted in South Africa during recent years resulted in the identification of 65 different LDLR gene mutations in ~50% of patients. The 65 LDLR gene mutations detected in the South African patients reflect ~10% of the total number of currently documented FH mutations. This emphasise the significance of these findings and provide a solid platform for subsequent mutation analysis. The relative low mutation detection efficiency, and non-segregation with markers at the LDLR and apo B genes in selected families, highlighted the value of DNA-based diagnostic methods in the complete genetic description of ADH. The information gained through the effort of defining the mutational spectrum in South Africa was applied to develop a cost-effective population-directed screening approach for accurate diagnosis of FH, a prerequisite for preventive treatment of this common lipid disorder. Evaluation of the types and frequencies of mutations identified have also broadened our understanding of disease patterns in the diverse South African population.

Table 1. Spectrum of mutations identified in the LDLR gene in different South African ethnic groups

Number Fig.1	Exon/ Intron	Molecular Event	Designation	CpG	No.	Origin/ Found in	References
AFRIKANER POPULATION							
7	Exon 3	T→G at 259	W66G	-	1	Fr-Canadian	Leitersdorf et al. 1990
11	Exon 4	G→A at 523	D154N	-	31	UK	Kotze et al. 1989b
13	Exon 4	A→G at 662	D200G	-	5	UK	Hobbs et al. 1992
59	Exon 4	Ins 18-bp after 681	681ins18	-	1	Germany	Kotze et al. 1996b
15	Exon 4	C→G at 681	D206E	+	144	UK	Kotze et al. 1990
21	Exon 6	C→T at 917	S285L	-	3	France	Hobbs et al. 1992
45	Exon 7	C→T at 1048	R329X	+	1	Norway	Solberg et al. 1994
65	Intron 7	T→C at 1061-8	IVS7-8T→C	-	1	Dutch	Jensen et al. 1996
22	Exon 8	G→A at 1130	C356Y	-	4	South Africa	This study
24	Exon 8	G→T at 1145	G361V	-	2	South Africa	This study
32	Exon 9	G→A at 1285	V408M	+	57	Netherland	Kotze et al. 1989b
39	Exon 15	C→T at 2177	T705I	-	1	Fr-American	Hobbs et al.1992
41	Exon 16	G→A at 2389	V776M	+	1	Cuba	Pereira et al. 1995
MIXED ANCESTRY (COLOURED POPULATION)							
1	Promoter	C→T at –59	-59c→t	-	1	South Africa	Scholtz 1999
3	Exon 1	T→C at 28	W-12R	-	1	Italian	Marx et al. 1997
4	Exon 2	G→A at 148	A29T	+	1	South Africa	Loubser et al. 1999
5	Exon 3	C→T at 232	R57C	+	1	English	Day et al. 1997b
6	Exon 3	C→T at 241	R60C	+	1	Danish	Nissen et al. 1998
52	Exon 4	del TC at 369	369delTC	-	1	South Africa	Loubser et al. 1999
11	Exon 4	G→A at 523	D154N	-	2	UK	Kotze et al. 1989b
54	Exon 4	del GGT at 652	652delGGT	-	4	Lithuania	Meiner et al. 1991

13	Exon 4	A→G at 662	D200G	-	1	UK	Hobbs et al. 1992
14	Exon 4	A→G at 680	D203A	-	1	South Africa	Loubser et al. 1999
15	Exon 4	C→ G at 681	D206E	+	19	UK	Kotze et al. 1990
16	Exon 4	G→A at 682	E207K	+	1	Fr-Canadian	Leitersdorf et al. 1990
17	Exon 4	T→G at 691	C210G	-	1	Norwegian	Sundvold et al. 1996
19	Exon 5	G→A at 772	E237K	-	1	South Africa	Loubser et al. 1999
64	Intron 6	G→A at 941-4	IVS6-4G→A	-	1	South Africa	Loubser et al. 1999
55	Intron 6-8	del 2.5-kb	del 2.5-kb	-	10	South Africa	Henderson et al. 1988
25	Exon 8	T→G at 1154	L364R	-	1	South Africa	Loubser et al. 1999
32	Exon 9	G→A at 1285	V408M	+	13	Netherland	Kotze et al. 1989b
37	Exon 14	C→T at 2054	P664L	+	3	India	Hobbs et al. 1992
BLACK POPULATION							
49	Promoter	del CTC at –92	-92delCTC	-	1	South Africa	Peeters et al. 1998
50	Exon 2	del 6-bp at 137	137del6	-	4	South Africa	Leitersdorf et al. 1988
51	Exon 2	del G at 172	172delG	-	1	South Africa	Hobbs et al. 1992
61	Intron 3	G→A at 313+1	IVS3+1G→A	+	1	South Africa	Thiart et al. submitted
10	Exon 4	G→C at 514	D151H	+	1	South Africa	Thiart et al. submitted
18	Exon 5	C→T at 756	R232W	+	1	South Africa	Thiart et al. submitted
28	Exon 9	G→A at 1217	R385Q	-	1	South Africa	Thiart et al. submitted
29	Exon 9	G→A at 1222	E387K	+	1	Algerian	Hobbs et al. 1992
38	Exon 14	C→T at 2096	P678L	+	1	German	Schuster et al. 1995
42	Exon 17	G→A at 2441	R793Q	+	1	South Africa	Thiart et al. submitted
INDIAN POPULATION							
2	Exon 1	A→T at 1	M-21L	-	1	South Africa	Langenhoven et al. 1996
5	Exon 3	C→T at 232	R57C	+	1	South Africa	Kotze et al. 1997
8	Exon 3	G→T at 268	D69Y	+	1	South Africa	Rubinsztein et al. 1993a
9	Exon 4	G→A at 418	E119K	+	1	USA	Rubinsztein et al. 1993a

12	Exon 4	G→T at 661	D200Y	+	1	Finland	Koivisto et al. 1995
16	Exon 4	G→A at 682	E207K	+	2	Fr-Canadian	Leitersdorf et al. 1990
47	Exon 8	C→A at 1175	C371X	-	1	South Africa	Langenhoven et al. 1996
27	Exon 9	C→G at 1215	N384K	-	1	South Africa	Kotze et al. 1997
37	Exon 14	C→T at 2054	P664L	+	10	India	Soutar et al. 1989
40	Exon 16	A→T at 2356	S765C	-	1	South Africa	Kotze et al. 1997
JEWISH POPULATION							
43	Exon 3	C→T at 253	Q64X	-	2	Germany	Schuster et al. 1995
44	Exon 4	C→T at 373	Q104X	-	1	South Africa	This study
54	Exon 4	del GGT at 652	652delGGT	-	5	Lithuania	Meiner et al. 1991
15	Exon 4	C→ G at 681	D206E	+	1	UK	Kotze et al. 1990
62	Intron 9	G→A at 1358+1	IVS9+1 G→A	-	1	South Africa	Callis (unpublished results)
31	Exon 9	C→G at 1284	N407K	+	1	South Africa	Callis (unpublished results)
32	Exon 9	G→A at 1285	V408M	+	1	Netherland	Kotze et al. 1989b
63	Intron 14	G→A at 2140+5	IVS14+5G→A	-	1	South Africa	Heath et al. 1999
EUROPEAN ANCESTRY							
5	Exon 3	C→T at 232	R57C	+	1	St H, Ind, Fr	Callis et al. 1998
58	Exon 4	ins G after 558	558insG	-	1	German	Thiart et al. 1998
53	Exon 4	del G at 617	617delG	-	1	French	Callis et al. 1998
15	Exon 4	C→ G at 681	D206E	+	1	UK	Kotze et al. 1990
20	Exon 6	G→A at 910	D283N	-	1	Irish	Bilheimer et al. 1985
23	Exon 8	A→C at 1133	Q357P	-	1	UK	Callis et al. 1998
46	Exon 8	C→T at 1150	Q363X	-	1	Cypriot	Kotze et al. 1997
26	Exon 8	C→G at 1156	D365E	-	1	Cypriot	Kotze et al. 1997
30	Exon 9	G→A at 1247	R395Q	+	1	German	Thiart et al. 1998
33	Exon 9	G→C at 1329	W422C	-	1	UK	Hobbs et al. 1992
60	Exon 10	Complex del/ins	1379del16→5bp	-	1	Wales	This study

34	Exon 10	T→C at 1447	W462R	-	1	Scotland	Ward et al. 1995
35	Exon 11	G→A at 1646	G528D	-	1	Greek	Hobbs et al. 1992
36	Exon 11	A→C at 1690	N543H	-	1	Netherlands	Tricot-Guerber et al. 1995
48	Exon 14	C→A at 2043	C660X	-	2	Lebanese	Lehrman et al. 1987
56	Exon 14	del T at 2092	2092delT	-	1	USA	Hobbs et al. 1992
63	Intron 14	G→A at 2140+5	IVS14+5G→A	-	1	Ireland	Heath et al. 1999
57	Exon 17	del 9-bp after 2393	2393del9-bp	-	1	Netherlands	Lombardi et al. 1996

Number refers to location of mutation, as illustrated in figure 1

Events occuring at a CpG dinucleotide: "+" indicates yes and "-" indicates no

Highlighted mutations indicate founder-related/common LDLR gene mutations

The majority of mutations summarised in this table were included in a recent mutation update (Varret et al. 1998)

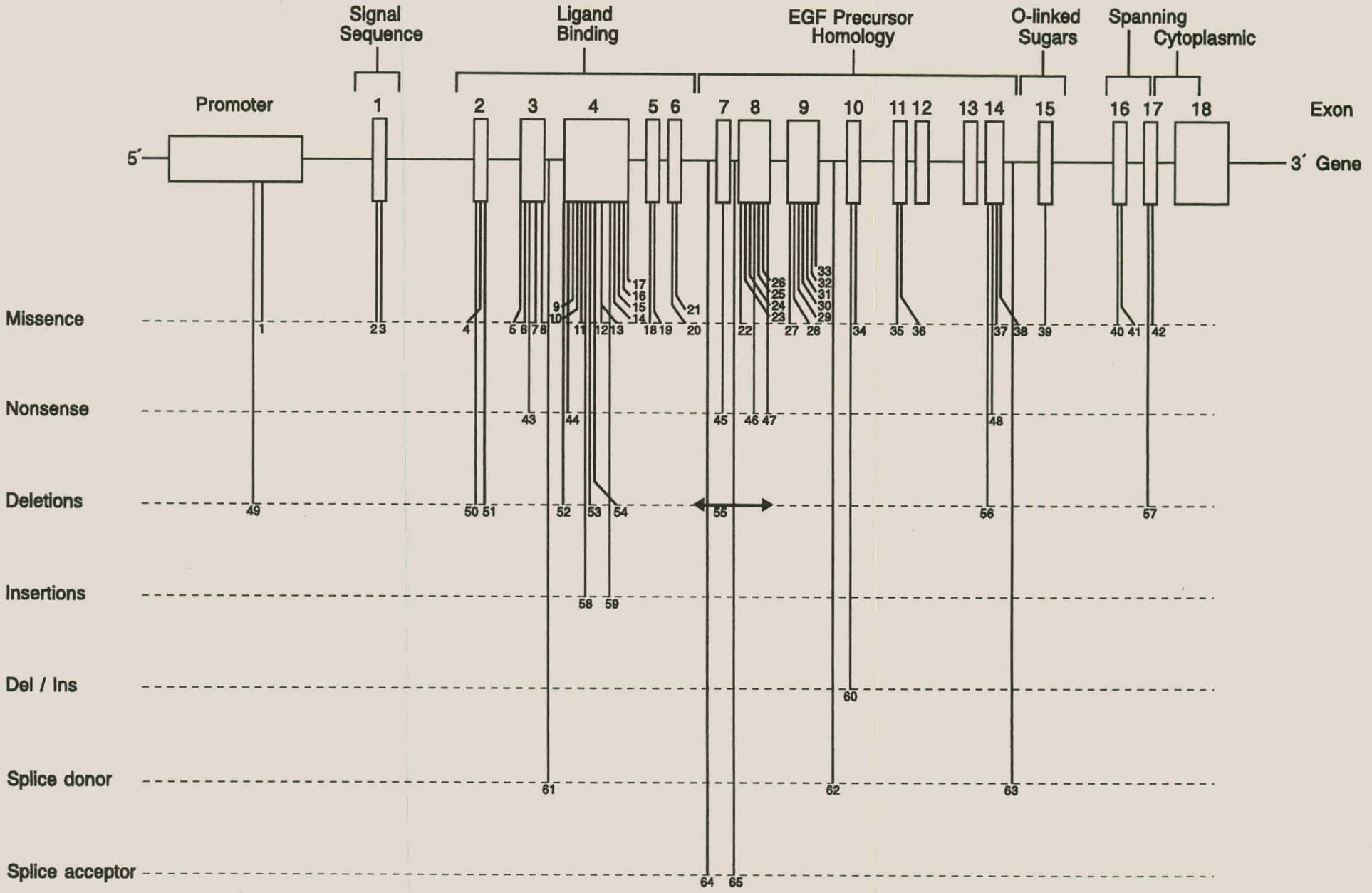
The possibility that some of the mutations listed here are not disease-causing, could not be excluded

Both mutations T705I (FH-Paris 9) and IVS7-8T→C are probably non-pathogenic (Jensen et al. 1996, Lombardi et al. 1997, Nissen et al. 1998).

"Afrikaner" refers to an individual of European descent, mainly Dutch, French, German and British stock; "Coloured" refers to an individual of mixed ancestry, including San, Khoi, African Negro, Madagascar, Javanese and European origin; "Black" refers to South Africans of central African descent.

Mutation names are given according to Beaudet et al. 1996 and Antonarakis et al. 1998.

Figure 1 Schematic representation of the genomic organisation of the LDLR gene in relation to five distinct functional domains of the LDLR gene. The sites of 65 different LDLR gene mutations identified in the South African population are shown. Further details of each mutation are provided in Table 1.



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Chapter 6

Conclusions

Inherited lipid disorders represent the most common form of genetic disease world-wide. Defects in the low density lipoprotein receptor (LDLR) and other lipid-related genes underlying atherosclerotic cardiovascular diseases, account for the majority of deaths in Western societies, with men usually affected 10 years earlier than women (Henderson 1996). Factors underlying population differences in coronary heart disease (CHD) mortality and morbidity have been under study for decades (Tunstall-Pedoe et al. 1994).

The treatable disorder, familial hypercholesterolemia (FH), has been well characterised in the South African Afrikaner population. Direct mutation screening for three founder-related LDLR gene mutations, D154N, D206E and V408M, causing FH in the majority of affected Afrikaners (Kotze et al. 1991), confirmed the high prevalence of heterozygous FH (~1/70) in this population (Steyn et al. 1997). Mutation analysis has now been extended to the remaining ~10% of Afrikaner FH patients who screened negative for the previously-described founder mutations, as well as several other homogeneous and heterogeneous populations. The purpose of this study was to define the mutational spectra in different populations, with the ultimate aim to compare the types and frequencies of mutations in an attempt to broaden our knowledge on disease patterns in these groups. In addition to providing the potential of accurate FH diagnosis in families, mutation screening revealed important information on the origin and distribution of LDLR gene mutations and contributed largely to our knowledge of the biological history of the South African population.

South African Population

Delineation of the mutational spectrum in the diverse South African population has provided some explanations for the observed ethnic differences in CHD risk, ranging from high to low in the Caucasian and Black populations, respectively, and intermediate in the Coloured population of mixed ancestry (Steyn et al. 1985). The exceedingly high death rate from premature CHD in Afrikaners is undoubtedly a consequence of multiple founder-related LDLR gene mutations underlying FH in this population of European descent. These include the well-characterised gene defects D154N, D206E and V408M accounting for FH in ~90% of affected Afrikaners (Kotze et al. 1991), and four apparently minor founder mutations D200G, S285L, C356Y and G361V. Haplotype analysis using intragenic LDLR gene polymorphisms and two highly informative microsatellite markers flanking the LDLR gene, were consistent with common origins for these mutations.

The founder-related basis for the high prevalence of FH in South Africa provided the opportunity to use direct mutation screening to test whether the increasing manifestation of CHD in the Coloured population (San, Khoi, African Negro, Madagascar, Javanese and European origin) can be explained by Caucasoid admixture. This appeared to be the case, since six founder-type "South African mutations" were responsible for FH in ~20% of the study cohort including 236 patients with primary hypercholesterolaemia (Loubser et al. 1999). Only one of these probands of mixed ancestry was heterozygous for mutation R3500Q causing familial defective apolipoprotein B-100 (FDB). Apart from the Afrikaner founder mutations, P664L and del197 known to be common in South Africa due to multiple entries of these mutant alleles into the Indian and Jewish communities, respectively, were also prevalent in the Coloured population. These findings indicated that Caucasoid admixture contribute significantly to the apparently high prevalence of FH in South Africans of mixed ancestry.

None of the LDLR gene defects identified in Black FH patients have been detected in any other South African population group. This finding is in accordance with data from Loubser et al. (1999), suggesting minimal, if any, admixture of the Black population with other populations. The unique mutational profile defined in the Black population of South Africa included mutations in the promoter and coding region of the LDLR gene, as well as a mutation in one of the splice junctions. Predominance of a 6-bp deletion in exon 2 of the LDLR gene (28%) in this population where FH is rare was an unexpected finding, that can probably be explained by earlier propagation of the mutant allele within a small group of people who later evolved separately into different African tribes. An interesting observation was the relatively mild expression of the 6-bp deletion in the homozygous state, confirming the initial hypothesis that the low prevalence and/or under diagnosis of FH in this population may be a consequence of altered expression of disease-related LDLR gene mutations due to interaction with other (protective) factors. Genetic variation was frequently detected in the LDLR promoter region in FH patients with an African genetic element, whilst apparently absent in South African Caucasians. This finding raises the possibility that sequence changes upstream of the gene may influence the phenotypic expression of the disease in populations where these mutations prevail (Scholtz et al. 1999).

Non-South African Populations

Knowledge on the molecular basis of FH in Costa Rica/South America and New Zealand is limited. FH patients from these populations included in this study were first screened for the founder-type mutation R3500Q (Soria et al. 1989) in the apo B gene, causing FDB in the majority of patients with this lipid disorder. This mutation was absent in the Costa Rican study cohort, but was identified in three of four New Zealand probands who demonstrated

normal receptor function using an *in vitro* lymphocyte receptor assay. The molecularly-uncharacterised hypercholesterolaemics were subsequently examined for LDLR mutations in the promoter and coding region by HEX-SSCP analysis, using three different gel systems. Four novel mutations (Q665X, I140T, 8-bp ins at 681 and 2389+6C→T) were identified in the Costa Rican study population (Kotze et al. 1996, Thiart et al. 1997, Varret et al. 1998/Appendix 2, Peeters et al. 1999). LDLR gene mutation screening in New Zealand patients led to the identification of a novel 2-bp deletion and several previously-described mutations including the Afrikaner-founder mutation, D206E (Theart et al. 1995, Thiart et al. submitted).

A comparison between the ratio of nonsense versus missense mutations detected in different populations (South Africans, New Zealanders and Costa Ricans), including those locally identified in Belgian FH patients (Varret et al. 1998/Appendix 2) indicated a ratio of 1/10 in South Africa to 1/5 in non-South African populations. The latter is in agreement with the findings of Day et al. (1997), who observed a fourfold overrepresentation of substitutional stop codons to amino acid changes; 1/5, compared to the expected value of 1/20 (Day et al. 1997, Hobbs et al. 1992). We therefore postulate that South Africa might be an especially good environment for unmasking "milder" LDLR mutations.

DNA diagnosis of FH: implications and new perspectives

Data generated during this study demonstrated that the array of mutations varies considerably in different populations. Increased knowledge of the mutational profile underlying FH in different countries/populations facilitated disease diagnosis, genetic counselling and preventive treatment in affected families. Failure to identify the disease-causing mutation in all the patients clinically diagnosed with FH, was initially ascribed to limitations imposed by

the mutation detection methods used. However, the low detection rate (~50%) and subsequent haplotype analysis performed at the LDLR and apo B gene loci, suggested the existence of additional genes underlying the FH phenotype. This hypothesis was recently confirmed by Varret et al. 1999/Appendix 3), implicating a third major locus for autosomal dominant hypercholesterolaemia (ADH) on chromosome 1p.

Future studies will be focussed on the elucidation of the molecular basis of ADH in mutation-negative subjects identified during this study. Both the homogeneous Afrikaner population and the heterogeneous Coloured population may serve as valuable candidate populations for future mapping studies. The latter could be applied in admixture linkage disequilibrium (ALD) approaches to map genes, while the Afrikaner population is useful because of the likely restricted number of founder mutations. In conclusion, this study has opened new avenues in the search for gene mutations underlying ADH.

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APPENDIX 1

Detailed Experimental Procedures

Protocol 1: Genomic DNA Extraction

Miller SA, Dykes DD, Polesky HF (1988) A simple salting out procedure for extracting DNA from human nucleated cells. Nucleic Acids Res 16:1215.

Lysis of tissues to cells

↓

Collect 5-10 ml blood in EDTA-tubes

Mix blood with 40 ml cold lysis buffer

Place on ice for 15 min.

Centrifuge for 10 min. at 1500 rpm

↓

Lysis of cells and isolation of nuclei

↓

Remove the supernatant

Wash cell pellet with 10 ml PBS buffer

Remove the supernatant

↓

Lysis of nuclei

↓

Re-suspend pellet in:

3 ml nuclear lysis buffer

50 µl 10 mg/ml proteinase K

300 µl 10% SDS

↓

Vortex for 5 seconds

Incubate overnight at 55°C

↓

Purification of DNA

↓

Add 1 ml 6 M NaCl solution to each sample

Shake vigorously for 1 min

Centrifuge for 15 min at 2500 rpm

Transfer the supernatant containing the DNA solution to a clean Falcon tube

↓

Precipitation of DNA

↓

Add 2X volume of cold absolute ethanol

Leave at room temperature

Transfer the precipitated DNA strands to a eppendorf tube

Wash DNA with 70% ethanol (to remove excess salt)

Centrifuge briefly

Remove excess ethanol

Air-dry the DNA at room temperature

Dissolve the DNA pellet in 300-500 µl SABAX-dH_2O

Protocol 2: Polymerase Chain Reaction (PCR)

Saiki RK, Scharf S, Faloona F, Mullis KB, Horn GT, Erlich HA, Arnheim N (1985) Enzymatic amplification of beta-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia. Science 230:1350-1354

Cocktail Mix

↓

20-50 ng genomic DNA

20 pmol each of forward and reverse primer

100 µM of each dNTP

10 X Buffer with 1.5 mM $MgCl_2$/ or without $MgCl_2$ +

appropriate amount of 25 mM $MgCl_2$

0.5 U Taq DNA polymerase (Boehringer Mannheim)

200 µM Cresol Red Loading Buffer

Nuclease-free water to a final volume of 25µl/50µl

↓

PCR amplification

↓

PERKIN ELMER GeneAmp 2400/9600 PCR System Thermal Cycler

Appropriate thermal cycling conditions

↓

Analysis PCR products

↓

Load 5 µl on a 2% Agarose gel

Electrophorese at 100 Volt for 20 min

Stain for 5 min in 1 X TBE containing EtBr

Examine gel with ultraviolet light illumination

Primers used for amplification

Primers for the promoter and coding region of the LDLR gene (Table 1)

Jensen, H. K., Jensen, L. G., Hansen, P. S., Faergeman, O. & Gregersen, N (1996) High sensitivity of the single-strand conformation polymorphism method for detecting sequence variations in the low-density lipoprotein receptor gene validated by DNA sequencing. Clinical Chemistry 42:1140-1146

Promotor Region

95 °C	5'	
95 °C	30''	} x 10 cycles
60 °C	45''	
72 °C	45''	
95 °C	30''	} x 30 cycles
58 °C	45'	
72 °C	45'	
35 °C	5'	

Coding Region

95 °C	5'	
94 °C	1'	
60/66 °C	2'	} x35 cycles
72 °C	10'	
25 °C	5'	

DGGE primers for the promoter and coding region of the LDLR gene (Table 2)

Nissen H, Guldberg P, Hansen AB, Petersen NE, Horder M (1996) Clinically applicable mutation screening in familial hypercholesterolemia. Hum Mutat 8: 168-177

95 °C	5'	
94 °C	1'	} x 40 cycles
66 °C	5'	
72 °C	10'	
99.9 °C	7'	
65 °C	50'	Ramp 10'
37 °C	50'	Ramp 10'
25 °C	10''	

*Top B, Uitterlinden AG, Van der Zee A, Kastelein JJP, Gevers Leuven JA, Havekes LM, Frants RR (1992) Absence of mutations in the promoter region of the low density lipoprotein receptor gene in a large number of familial hypercholesterolaemia patients as revealed by denaturing gradient gel electrophoresis. Hum Genet 89:561-565

Primers for the Multiplex PCR assay (Table 3)

Kotze MJ, Theart L, Callis M, Peeters AV, Thiart R, Langenhoven E (1995) Nonradioactive-multiplex PCR screening strategy for the simultaneous detection of multiple low-density lipoprotein receptor gene mutation. PCR Methods and Applications 4: 352-356

94 °C	5'	
94 °C	1'	x 15 cycles
62 °C	1'	
72 °C	2'	
94 °C	1'	x 20 cycles
55 °C	1'	
72 °C	2'	
35 °C	10''	

Primers for Microsatellite Analysis (Table 4)

Traeger-Synodinos J, Mavroidis N, Kanavakis E, Drogari E, Humphries SE, Day IN, Kattamis C, Matsaniotis N (1998) Analysis of low density lipoprotein receptor gene mutations and

microsatellite haplotypes in Greek FH heterozygous children: six independent ancestors account for 60% of probands. Hum Genet 102:343-347

Marker D19S221

95 °C	5'		
94 °C	15''	}	x 30 cycles
62 °C	15''		
72 °C	30''		
72 °C	10'		
25 °C	5'		

Marker D19S394

94 °C	2'		
94 °C	1'	}	x 5 cycles
59 °C	1'		
72 °C	2'		
94 °C	1'	}	x 30 cycles
55 °C	1'		
72 °C	2'		
33 °C	10''		

Table 1. Primers used to amplify the promoter and coding region of the LDLR gene

Region/ Exon	Oligonucleotide sequence (5' to 3')	Fragment size (bp)
Annealing temperature of 58 °C		
Promoter	F GAGGCAGAGAGGACAATGGC R CACGACCTGCTGTGTCCAAGCTTGAAACCC	277
Annealing temperature of 60 °C		
1	F CACATTGAAATGCTGTAAATGACG R CTATTCTGGCGCCTGGAGCAAGCC	215
2	F TTGAGAGACCCTTTCTCCTTTTCC R GCATATCATGCCCAAAGGGG	183
5	F AGAAAATCAACACACTCTGTCCTG R GGAAAACCAGATGGCCAGCG	180
6	F TCCTCCTTCCTCTCTCTGGC R TCTGCAAGCCGCCTGCACCG	179
10	F ATGCCCTTCTCTCCTCCTGC R AGCCCTCAGCGTCGTGGATA	278
12	F ACTGGCATCAGCACGTGACC R CGTGTGTCTATCCGGCCACC	236
15	F AGAAGACGTTTATTTATTCTTTC R GTGTGGTGGCGGGCCCAGTCTTT	217
16	F CCTTCCTTTAGACCTGGGCC R CATAGCGGGAGGCTGTGACC	173
Annealing temperature of 66 °C		
3	F TTCCTTTGAGTGACAGTTCAATCC R GATAGGCTCAATAGCAAAGGCAGG	196
4A	F GTGGTCTCGGCCATCCATCC R AGCCATCTTCGCAGTCGGGG	242
4B	F CCCCCAGCTGTGGGCCTGCG R CGCCCCCACCCTGCCCCGCC	237
7	F GGCGAAGGGATGGGTAGGGG R GTTGCCATGTCAGGAAGCGC	236
8	F CATTGGGGAAGAGCCTCCCC R GCCTGCAAGGGGTGAGGCCG	220
9	F CCCCTGACCTCGCTCCCCGG R GCTGCAGGCAGGGGCGACGC	224
11	F TCCTCCCCCGCCCTCCAGCC R GCTGGGACGGCTGTCCTGCG	194
13	F GTCATCTTCCTTGCTGCCTG R TTCCACAAGGAGGTTTCAAGGTTGGGGGGG	329
14	F AAATTTCTGGAATCTTCTGG R GCAGAGAGAGGCTCAGGAGG	268
17	F GGGTCTCTGGTCTCGGGGGC R GGCTCTGGCTTTCTAGAGAGGG	242
18	F GCCTGTTTCCTGAGTGCTGG R TCTCAGGAAGGGTTCTGGGC	135

F = Forward primer, R = Reverse primer

Table 2. DGGE primers used to amplify the promoter and coding region of the LDLR gene

Region/ Exon	Oligonucleotide sequence (5' to 3')	Fragment size (bp)
30% to 70% denaturant gradient		
Promoter*	F 40-bp GC-clamp-AGGACTGGAGTGGGAATCAGAGC R TGCTGTGTCCTAGCTGGAAACCC	252
2	F 40-bp GC-clamp-CGTGGTCAGTTTCTGATTCTGGCG R ATAAATGCATATCATGCCCAAAGG	253
3	F 40-bp GC-clamp-TCGGCCTCAGTGGGTCTTTC R ACTCCCCAGGACTCAGATAGGC	268
5	F 40-bp GC-clamp-GGCCCTGCTTGTTTTTCTCTGG R AGCAGCAAGGCACAGAGAATGG	282
6	F 40-bp GC-clamp-ACGAAACTGAGGCTCAGACACACC R GCTCCCCACAAACTCTGCAAGC	262
10	F GCAGTGAGATGAGGGCTCCTGG R 40-bp GC-clamp-CCTGCAGCCCTCAGCGTCG	349
11	F 40-bp GC-clamp-GGATCCTCCCCCGCCCTC R TGGCTGGGACGGCTGTCC	239
12	F GGCCCTCAGGCCCTCTGG R 40-bp GC-clamp-CCGAGTTTTCTGCGTTCATCTT	336
13	F 50-bp GC-clamp-GTCATCTTCCTTGCTGCCTG R CACAAGGAGGTTTCAAGGTTGG	264
17	F 50-bp GC-clamp-GGGCAGCTGTGTGACAGAGCG R CATGGCTCTGGCTTTCTAGAGAGG	279
ApoB**	F 40-bp GC-clamp-GGAGCAGTTGACCACAAGCTTAGC R GGTGGCTTTGCTTGTATGTTCTCC	382
40% to 80% denaturant gradient		
1	F 50-bp GC-clamp-TTGAAATGCTGTAAATGACGTGG R CTGGCGCCTGGAGCAAGC	256
4A	F 40-bp GC-clamp-ACTGCGGCAGCGTCCCCGGC R GGATGCAGGTGGAGCTGTTGC	297
4B***	F ACCTGTGGTCCCGCCAGC R 40-bp GC-clamp-CCAGGGACAGGTGATAGGACG	345
7	F 40-bp GC-clamp-AGAGTGACCAGTCTGCATCCCTGG R TTGGTTGCCATGTCAGGAAGC	253
8	F 40-bp GC-clamp-TCCCCACCAAGCCTCTTTCTCTC R CCACCCGCCGCCTTCC	222
9	F 46-bp GC-/10-bp AT-clamp-CTGACCTCGCTCCCCGGACC R GGCTGCAGGCAGGGGCGACG	278
14	F 50-bp GC-clamp-TCTCGTTCCTGCCCTGACTCC R GACACAGGACGCAGAAACAAGG	274
15***	F 3-bp GC-clamp-GGCACGTGGCACTCAGAAGACG R 50-bp GC-clamp-GTGTGGTGGCGGGCCCAGTCTTT	288
16	F 50-bp GC-clamp-CTCCATTTCTTGGTGGCCTTCC R CATAGCGGGAGGCTGTGACCTGG	239
18	F 50-bp GC-clamp-CCTGAGTGCTGGACTGATAGTTTCC R AAGGCCGGCGAGGTCTCAGG	190

F = Forward primer, R = Reverse primer

* see also promoter primers that span the -175 region: Top et al. (1992)

**Apolipoproteien B-100 (apoB) gene

***Require 25 mM MgCl2 in PCR mixture

3-bp GC-clamp: CGG

40-bp GC-clamp: CGCCCGCCGCGCCCCGCGCCCGTCCCGCCGCCCCCGCCCG

46-bp GC-/10-bp AT-clamp: CGCCCGCGCCCGCCGCGCCCCGCGCCCGTCCCG-CCGCCCCCGCCCGAAATAATAAA

50-bp GC-clamp: CGCCCGCCGCCGCCCGCCGCGCCCCGCGCCCGTCCCGCC GCCCCCGCCCG

Table 3. Multiplex amplification refractory mutation system (ARMS) primers used for amplification of the LDLR gene

Exon	Oligonucleotide sequence (5' to 3')
4	F (4.2.1) CGAGGCCTCCTGCCCGGTGCTCACC R (4.5.3) GGGCCTGCGACAACGACCCCGACTGCGAAA
4	F (U) GGGACCCAGGGACAGGTGATAGGAC R (4.7) CCCGCCCATACCGCAGTTTTCCTCC
9	F (9.5) GCTCACCTGCAGATCATTCTCTGGG R (9.7) AGCCTCATCCCCAACCTGAGGACCA
ApoB*	F GGAGCAGTTGACCACAAGCTTAGCTTGGAA R TGGAAGTGCCCTGCAGCTTCACTGAAGAAT

F = Forward primer, R = Reverse primer
*Mutation R3500Q in the Apoliprotein B-100 (ApoB) gene
PCR reaction requires 2mM tetramethylammonium chloride (Me4NCl) and 15% glycerol

Table 4. Microsatellite primers for haplotype associations

Marker	Oligonucleotide sequence (5' to 3')
D19S221	F FAM-GCAAGACTCTGACTCAACAAAA R PIG-tail-CATAGAGATCAATGGCA
D19S394	F* AGACTACAGTGAGCTGTGG R GTGTTCCTAACTACCAGGC

F = Forward primer, R = Reverse primer
*Can add FAM-labelling to 5'-primer
PIG-tail: GTTTCTT

Protocol 3: Heteroduplex-Single-Strand Conformation Polymorphism (HEX-SSCP) Analysis

Kotze MJ, Theart L, Callis M, Peeters AV, Thiart R, Langenhoven E (1995) Nonradioactive multiplex PCR screening strategy for the simultaneous detection of multiple low-density lipoprotein receptor gene mutation. PCR Methods and Applications 4:352-356

Gel Preparation

↓

Clamp the two glass plates (8 x 20 cm) together with binder clips

Pour an agarose plug

Pour polyacrylamide gel between the two glass plates (1 mm)

Gel polymerise for ~10-15 minutes

↓

Electrophoresis

↓

Load 10-15 µl PCR product (PCR was performed with cresol-red loading buffer)

or

10 µl PCR product mixed with 5µl bromophenol-blue loading buffer + 5µl TBE

Denature for 5 min. at 90-95°C

Place on ice

Load samples in a 10% polyacrylamide gel supplemented with Urea or Gliserol and on a 20% polyacrylamide gel (Mighty Small)

Electrophorese at room temperature overnight at 250 Volt

↓

Staining of polyacrylamide gel

↓

Stain gel in 1 X TBE containing 100µg/ml EtBr for 10 min

Destain gel in dH_2O or 1 X TBE

Examine with ultraviolet light illumination

Protocol 4: Denaturing Gradient Gel Electrophoresis (DGGE)

Nissen H, Guldberg P, Hansen AB, Petersen NE, Horder M (1996) Clinically applicable mutation screening in familial hypercholesterolemia. Hum Mutat 8:168-177

Gel Preparation:

Denaturant concentrations: 30-70% and 40-80%

↓

Clamp two glass plates (8 X 20 cm), with 1 mm spacers, together with binder clips

(A "homemade" gel apparatus are currently in use in our laboratory)

↓

Place the gradient mixer above the DGGE frame

Pour 14 ml of the solution with the **L**ower denaturant concentration (eg. 30%) in the **L**eft and 14 ml of the higher concentration (eg. 70%) in the right chamber of the gradient mixer.

↓

Open the connection between the two chambers and let the gel-solution pass through the plastic tubing between the two glass plates (Avoid air bubbles).

Leave gel to polymerise for 60 min.

↓

Electrophoresis

↓

Place the gel apparatus into the bath containing 1 X TAE buffer heated to 60 °C.

Load 7.5-10 µl PCR product on the gel

(PCR was performed in cresol-red loading buffer)

Electrophorese for 5 hours at 150 Volt or overnight at 60 Volt

↓

Staining

↓

Stain gel in 1 X TAE containing EtBr

Examine gel with ultraviolet light illumination

Protocol 5: DNA Sequencing

Direct DNA Sequencing

T7 Sequenase Version 2.0 Kit (USB)

Enzymatic Pre-treatment of PCR product

↓

5-7 µl PCR amplification mixture (purified product)

1 µl Exonuclease I (10U/µl)

(removes residual single stranded primers and extraneous DNA products)

1 µl Shrimp Alkaline Phosphatase (2U/µl)

(removes remaining dNTP's)

↓

Incubate at 37°C for 15 minutes

Incubate at 80 °C for 15 minutes (inactivates Exonuclease I and SAP)

↓

Annealing DNA and primer

↓

5 µl enzyme-treated PCR product

Primer (5-10 pmol/µl)

dH_2O to 10 µl

↓

Denature at 100°C for 3 min

Place on ice for 5 min

↓

Labelling Reaction

↓

10 µl cold annealed DNA mixture

2 µl reaction buffer

1 µl 0.1M DTT

2 µl diluted labelling mix (1:5)

0.5 µl ^{35}S dATP

2 µl Sequenase enzyme

↓

Place on ice for 10 min

↓

Termination Reaction

↓

Add 2.5 µl of each (G, A, T, C) termination mix in four separate eppendorf tubes

Incubate at 37°C for 5 min

Add 3.5 µl labelling reaction to each termination tube

Incubate at 37°C for 10 min

Add 4 µl stop solution to terminate the extention reaction

↓

Prepare samples for loading

↓

Heat the samples at 75-80 °C for 2 minutes

Put on ice immediately

↓

Set up the sequencing run

↓

6% polyacrylamide sequencing gel polymerise for 2-24 hours

Wash gel slots well to remove excess urea, etc

Preheat gel for at least 30 min to 47-50 °C

Load 2 µl on the gel.

Electrophorese at 47-50 °C at constant voltage (2500 Volt) for 1-3 hours

↓

Gel drying and exposure

↓

Fix gel in Acetic acid:Methanol (1:3) for 10-15 min

Transfer gel to filter paper and cover with cling wrap

Dry gel for 30 min on gel drying apparatus

Autoradiograph overnight at room termperature

Automated Fluorescent DNA Sequencing

ABI 373A Stretch automated sequencer

Enzymatic Pre-treatment of PCR product

↓

3 µl PCR amplification mixture (purified product)

0.5 µl Exonuclease I (10U/µl)

(removes residual single stranded primers and extraneous DNA products)

0.5 µl Shrimp Alkaline Phosphatase (2U/µl)

(removes remaining dNTP's)

↓

Incubate at 37 °C for 15 minutes

Incubate at 80 °C for 15 minutes (inactivate Exonuclease I and SAP)

↓

Cycle Sequencing

↓

4 µl enzyme-treated PCR product

4 µl Terminator Ready Reaction Mix (available from kit)

Primer (5-12 pmol/µl)

dH_2O to 20 µl

↓

2400 PE cycle program

I) 94 °C 2'

II) 94 °C 20''
59 °C 20'' (primer Tm)
60 °C 30''
(X 30)

III) 72 °C 5'
25 °C 10'

↓

Precipitation

↓

Transfer the 20 µl PCR sequencing reaction to a 1.5 ml microcentrifuge tube

Add 20 µl 2mM MgCl

And 50 µl 95% EtOH

↓

Mix and leave 10-15 minutes at room temperature

Centrifuge for 15-20 minutes

Dry pellet

↓

Prepare samples for loading

↓

Add 4 µl of the loading buffer to the pellet

Vortex/spin

Heat the samples at 90 °C for 2-5 minutes

Immediately put them on ice

↓

Set up the sequencing run

↓

CLEAN the glass plates with Alconox (3-4 times)

leave to air-dry

Leave gel to polymerise for 2-24 hours (Preferable for 2 hours)

Place the gel in the 373A machine and fill buffer chambers with 1 X TBE buffer

Load 2 µl (Sample pellet + 4 µl loading buffer) on the gel.

Electrophorese overnight for 14 hours (only for ABI 373)

Analyse on the 373A system

Protocol 6: Restriction Enzyme Digestion (RED)

Sambrook J, Fritsch EF and Maniatis T (1989) in Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory Press NY Vol 1, 2 and 3.

RED preparation

↓

10 µl PCR product (~40 ng)

2 µl 10 X Enzyme-specific buffer (1/10 of total volume)

3-10 Units restriction enzyme

dH_2O to 20 µl

↓

Incubation

↓

Incubate at appropriate temperature for 3 hours or overnight

Add 5-10 µl Bromophenol blue- or Ficoll-Orange G loading buffer

↓

Analyse RED products

↓

Load on an Agarose or Polyacrylamide gel

(depending on fragment size)

Stain for 5-10 min in 1 X TBE containing EtBr

Examine gel with ultraviolet light illumination

Protocol 7: Haplotype Association/Analysis

Direct Microsatellite Analysis

Sambrook J, Fritsch EF, Maniatis T (1989) Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

End-labelling of Primer

↓

10 pmol primer

5 µl γ^{32}P dATP (100 µCi)

16 U T4-polynucleotide-kinase (PNK)

PNK-Buffer

dH_2O to 20 µl

↓

Incubate at 37°C for 45 min

Inactivate at 70°C for 10 min

↓

Preparation for loading

↓

PCR with 1 pmol End-labelled primer per PCR

↓

4 µl PCR product (γ^{32}P dATP end-labelled primer)

Add 4 µl of the Bromophenol Blue loading buffer

Heat the samples at 95 °C for 5-10 minutes

Put on ice immediately

↓

Set up the sequencing run

↓

6% denaturing polyacrylamide gel, left to polymerise for 2-24 hours

Load 2.5 µl on the gel

Load a α^{35}S-labelled M13-DNA marker

Electrophorese at 47-50 °C and constant voltage for 2200 Volt for 3 h

↓

Gel drying and exposure

↓

Fix in Acetic acid:Methanol (1:3) for 10-15 min

Transfer gel to filter paper and cover with cling wrap

Dry gel for 30 min on gel drying apparatus

Autoradiograph overnight at room temperature

Automated Fluorescence Genescan Analysis

ABI A373 Stretch automated sequencer from Perkin Elmer

GENESCAN 672

Fluorescence PCR product preparation for loading

↓

0.5 µl PCR product (FAM-labelled primer)

Add 4 µl of the Blue Dextran loading buffer

Add 0.5 µl internal size standard Genescan ROX 500 marker

Vortex/spin

Heat the samples at 95 °C for 5-10 minutes

Put on ice immediately

↓

Set up the polyacrylamide run

↓

CLEAN the glass plates with Alconox (3-4 times)

leave to air-dry

Leave gel to polymerise for 2-24 hours (Preferable for 2 hours)

Plac gel in the 373A machine and fill buffer chambers with 1 X TBE buffer

Load 2 µl on the gel.

Electrophorese overnight for 9 hours (only for ABI 373)

Analyse on the 373A system: GENESCAN 672

Solutions

40% Acrylamide (5%C) stock

30 g BIORAD Bis-acrylamide:Acrylamide [19:1]

dH_2O to 75 ml

40% Acrylamide (5%C) stock

76 g Acrylamide

4 g Bis-acrylamide

dH_2O to 200 ml

40% Acrylamide (1%C) stock

39.6 g Acrylamide

0.4 g Bis-acrylamide

dH_2O to 100 ml

30% Acrylamide (3.4%C) stock

58.8 g Acrylamide

1.2 g Bis-acrylamide

dH_2O to 200 ml

2% Agarose gel

2 g Seakem LE Agarose

20 ml 5 X TBE

dH_2O to 100 ml

10% Ammonium persulfate (APS)

1 g Ammonium persulfate

dH_2O to 10 ml

Blue Dextran Loading buffer (for automated sequencing/genescan)

5 ml 50 mM EDTA (pH 8.0)

150 mg Blue Dextran (30 mg/ml)

25 ml deionised formamide

Bromophenol Blue loading buffer

0.0125 g 0.05% Xylene cyanol FF

0.0125 g 0.05% Bromophenol blue

0.168 g 20 mM EDTA (disodiumsalt)

22.7 ml deionised formamide

dH_2O to 25 ml

Cresol Red loading buffer

100 ml Cresol Red (1 mmol/l)

60 g Sucrose

0% Denaturant stock solution, (6% Acrylamide)

75 ml 40% Acrylamide

10 ml 50 X TAE

dH_2O to 500 ml

80% Denaturant stock solution, (6% Acrylamide)

75 ml 40% Acrylamide

10 ml 50 X TAE

169 g Urea

160 ml Formamide

dH_2O to 500 ml

30% Denaturant gradient	70% Denaturant gradient
8.75 ml 0% denaturant	1.75 ml 0% denaturant
5.25 ml 80% denaturant	12.25 ml 80% denaturant
14.0 ml	14.0 ml

Add in both: 7.2 µl TEMED

125 µl APS

40% Denaturant gradient	80% Denaturant gradient
7 ml 0% denaturant	14 ml 80% denaturant
ml 80% denaturant	
14 ml	

Add in both: 7.2 µl TEMED

125 µl APS

0.5 M EDTA, pH 8.0

146.1 g EDTA

dH_2O to 1 liter

Adjust pH 8.0 with NaOH

10 mg/ml Ethidium Bromide

1 g EtBr (10 mg/ml) in 100 ml H_2O

Ficoll-Orange G loading buffer

0.1 g (m/v) Orange G (final 0.1%)

20 g (m/v) Ficoll (final 20%)

0.29 g EDTA (pH 7.0) (final 10mM)

dH_2O to 100 ml

Lysis buffer

8.3 g NH_4Cl (final 155 mM)

1.1 g $KHCO_3$ (final 10 mM)

0.03 g EDTA (pH 7.4) (final 0.1 mM)

dH_2O to 1 liter

Nuclei lysis buffer

1.21 g Tris-Cl (final 10 mM)

23.4 g NaCl (final 400 mM)

0.6 g EDTA (pH 8.2) (final 2 mM)

dH_2O to 1 liter

PBS buffer

Dissolve 10 tablets in 1 liter ddH_2O

6% Polyacrylamide (for automated sequencing)

25 g Urea

1 g Amberlite

7.5 ml 40% Acrylamide

18 ml dH_2O

Mix well, filter and degas for 10 minutes

Add 5 ml 10 X TBE

dH_2O to 50 ml

Add 300 µl 10% APS

16 µl TEMED

6% Polyacrylamide sequencing gel

37.5 ml 40% (5%C) acrylamide

120 g Urea

50 ml 5 X TBE

dH_2O to 250 ml

6% Polyacrylamide (Sequencing gel)

37.5 ml 40% (5%C) acrylamide

120 g Urea

50 ml 5 X TBE

dH_2O to 250 ml

10% Polyacrylamide gel (RED)

2.5 ml 40% (3.4%C) acrylamide

2 ml 5 X TBE

5.39 dH_2O

80 µl 10% APS

30 µl TEMED

10% Polyacrylamide gel supplemented with 5% Glycerol (HEX-SSCP)

3 ml Glycerol

15 ml 40% acrylamide (1%C) stock

6 ml 5 X TBE (0.5 X)

36 ml ddH_2O

800 µl 10% APS

80 µl TEMED

10% Polyacrylamide gel supplemented with 7.5% Urea (HEX-SSCP)

4.5 g Urea

15 ml 40% acrylamide (1%C) stock

18 ml 5 X TBE (1.5 X)

26 ml ddH_2O

800 µl 10% APS

80 µl TEMED

20% Polyacrylamide gel on Mighty Small (SSCP)

5 ml 40% acrylamide (1%C) stock

1 ml 5 X TBE (0.5 X)

4 ml ddH_2O

80 µl 10% APS

40 µl TEMED

10 mg/ml Proteinase K

10mg Proteinase K

SABAX H_2O to 1 ml

50 X TAE electrophoresis buffer 5

242 g Tris base

57.1 ml glacial acetic acid

100 ml 0.5 M EDTA, pH 8.0

dH_2O to 1 liter

10 X TBE electrophoresis buffer (for automated sequencing)

108 g Tris base

55 g Boric acid

8.3 g Na_2 EDTA

dH_2O to 1 liter

APPENDIX 2

LDLR Database (second edition): new additions to the database and the software, and results of the first molecular analysis

Varret M, Rabés JP, Thiart R, Kotze MJ, Baron H, Cenarro A, Descamps O, Ebhardt M, Hondelijn JC, Kostner GM, Miyake Y, Pocovi M, Schmidt H, Schmidt H, Schuster H, Stuhrmann M, Yamamura T, Junien C, Béroud C, Boileau C

Nucleic Acids Research (1998) 26:248-252

248–252 Nucleic Acids Research, 1998, Vol. 26, No. 1

LDLR Database (second edition): new additions to the database and the software, and results of the first molecular analysis

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ABSTRACT

Mutations in the LDL receptor gene (LDLR) cause familial hypercholesterolemia (FH), a common autosomal dominant disorder. The LDLR database is a computerized tool that has been developed to provide tools to analyse the numerous mutations that have been identified in the LDLR gene. The second version of the LDLR database contains 140 new entries and the software has been modified to accommodate four new routines. The analysis of the updated data (350 mutations) gives the following informations: (i) 63% of the mutations are missense, and only 20% occur in CpG dinucleotides; (ii) although the mutations are widely distributed throughout the gene, there is an excess of mutations in exons 4 and 9, and a deficit in exons 13 and 15; (iii) the analysis of the distribution of mutations located within the ligand-binding domain shows that 74% of the mutations in this domain affect a conserved amino-acid, and that they are mostly confined in the C-terminal region of the repeats. Conversely, the same analysis in the EGF-like domain shows that 64% of the mutations in this domain affect a non-conserved amino-acid, and, that they are mostly confined in the N-terminal half of the repeats. The database is now accessible on the World Wide Web at http://www.umd.necker.fr

THE LDL RECEPTOR AND HYPERCHOLESTEROLEMIA

The LDL receptor is a 160 kDa transmembrane glycoprotein ubiquitously distributed, playing a major role in cholesterol homeostasis (1). Impairement of LDL receptor activity results in the accumulation of LDL cholesterol in the circulation leading to familial hypercholesterolemia (FH). Affected individuals display arcus corneae, tendon xanthomas and premature symptomatic coronary heart disease (2). FH is an autosomal dominant disease, homozygotes being more severely affected than heterozygotes. FH is also one of the most common inherited disorders with frequencies of heterozygotes and homozygotes estimated to be 1/500 and $1/10^6$, respectively. In certain communities FH frequency is higher due to founder effects (3). The LDL receptor gene (LDLR) lies on the short arm of chromosome 19 (19p13.1–13.3) (4,5). It contains 18 exons encoding the six functional domains of the mature protein: Signal peptide, ligand-binding domain, epidermal growth factor (EGF) precursor like, O-linked sugar, transmenbrane and cytoplasmic (6). To date,

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444 mutations in the LDLR gene have been identified that are distributed as follows: 350 point mutations (77%), 68 major rearrangements (15%), 20 splice mutations (4%), 6 mutations in the promoter sequence (1%) (3,7).

THE LDLR DATABASE

This second version of the LDLR database contains 350 entries. Table 1 shows the 140 new entries of the database corresponding to mutations either recently published or contributed by the co-authors of this paper (8–31). It is not intended to replace primary publications, although it does contain unpublished data. As in the previous edition, mutation names are given according to Beaudet *et al.* (32) and are often followed by the name of the city or country from which the proband's family originated. For each mutation, information is provided at several levels: gene (exon and codon number, wild type and mutant codon, mutational event, mutation name), protein (wild type and mutant amino acid, affected domain, activity, mutation class), personal (ethnic background, age, sex, body mass index, familial history of coronary heart disease), clinical (values of plasma total cholesterol, LDL-cholesterol, HDL-cholesterol and triglycerids, presence or absence of xanthomas, arcus corneae and symptomatic coronary heart disease) and impact (private, recurrent, founder). We have included possible recurrent mutations (when no comparable haplotypes of the LDLR gene where available) in two instances: (i) when carriers of the same mutation were from distant ethnic or geographic background, and if not (ii) when clinical data were provided for the mutations to allow analysis of phenotypic variability. This last point concerns mutations W23X identified in probands of German–Canadian and German origin, 533ins8 and R395Q identified in probands from Germany, D200G identified in probands of Afrikaner and British origin, S285L identified in probands of Afrikaner and Dutch origin and P664L identified in probands of Belgian, Flemish–Walloon and Dutch origin. The ambiguity between recurrent and founder mutations will only be solved when a consensus will be reached on the polymorphic sites of the LDLR gene that should be systematically typed. Finally, since many teams now systematically screen the whole gene, two-mutations alleles are now being reported. Eleven of these appear in Table 2 (18,33–37). They are not included in the mutations file of the database since it cannot, at present, accomodate two mutations on a single allele.

NEWLY DEVELOPED SOFTWARE ROUTINES

The software package contains routines for the analysis of the LDLR database that were developed with the 4th dimension^R (4D) package from ABI. The purpose of the software is to facilitate the mutational analysis of the LDLR gene at the molecular level and to provide the tools to promote the analysis of relationships between phenotype and genotype. Initially, six specific routines were developed (3). Four new routines have been added to the software: (i) «Restriction enzyme» appears on the first page of the mutation record. If the mutation modifies a restriction site, the program shows a restriction map displaying the new or abolished site and the enzymes of interest (Table 1, Column I). (ii) «Amino acid type search» studies the mutations with respect to phylogenic conservation. In effect, the LDLR gene has been identified, sequenced and converted to protein sequence in four mammalian species [complete coding sequence of the chinese hamster (SWISS-PROT accession number: p35950), the rabbit (p20063), the rat (p35952) and the mouse (p35951) LDL receptor] and in the xenope (38). The identity at the amino acid level between the human and chinese hamster, rabbit, rat, mouse and xenopus sequences are 81%, 79%, 77%, 76% and 70%, respectively. Therefore, the routine lists the mutations affecting conserved or non-conserved amino acids in the four mammals, in the xenope, or in all these sequences. (iii) «Phylogeny» studies the distibution of mutations (missense, stop and frameshift) in conserved amino acids between humans and mammals or vetebrates and in amino acids specifically found in the human protein. (iv) «Binary comparison» compares two mutation groups, each group being defined by distinct research criteria chosen from the database records (molecular, clinical, personal, etc.). The result can be displayed as either of several graphic representations (by amino acids, by exon, or by protein domain) of the distribution of the sorted mutations. Furthermore, the sorted mutations can also appear in a cumulated or detailed format (insertion, deletion, missense, nonsense).

RESULTS OF THE FIRST MOLECULAR ANALYSIS

The results of the first molecular analysis of the 350 point mutations of the database shows that 63% of the mutations are missense, and only 20% occur in CpG dinucleotides in opposition to the 32% observed in other human disease genes (39). The origin of this deficit is unknown. Although the mutations are widely distributed throughout the gene, there is an excess of mutations in exon 4 ($P = 0.001$) coding for the three central repeats of the ligand binding domain, and in exon 9 ($P = 0.01$) coding for the NH_2 end of the central region of the EGF precursor like domain, between repeats B and C. Conversely, there is a deficit of mutations in exon 13 ($P = 0.001$) coding for the COOH end of the central region of the EGF precursor like domain, between repeats B and C, and in exon 15 ($P = 0.001$) coding for the O-linked sugar domain. These mutation hot- or cold-spots cannot be attributed to a technological bias since most teams screened the 18 exons of the LDLR gene. The analysis of the distribution of mutations in the ligand-binding domain, after alignment of the seven repeats, shows that 74% of the mutations in this domain affect a conserved amino acid, and that they are mostly located in the C-terminal region of the repeats. Conversely, the same analysis in the EGF-like domain, after alignment of the three repeats, shows that 64% of the mutations in this domain affect a non-conserved amino acid, and that they are mostly clustered in the N-terminal half of the repeats. Finally, the investigation of genotype/phenotype correlations remains difficult since clinical data are usually incomplete in many published mutation reports. Furthermore, many mutations were identified in compound heterozygotes and the clinical data provided results from the combined effect of the two mutations. To overcome this shortage, we are currently developing an entry in the Web site that will facilitate the input of high quality clinical information for each mutation.

DATABASE ON THE WEB

The LDLR database is now accessible through the World Wide Web at http://www.umd.necker.fr . Users of the database must cite this article. Finally, notification of omissions and errors in the

Table 1. The 140 new mutation reports of the LDLR database

A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T
213	1	1	-21	ATG	TTG	A->T	No	Nla III -	M-21L	Met	Leu	SP	1	Htz	Wa		P	S. Afr. Indian	26
358	2	91	10	GAG	TAG	G->T	No	Rma I +	E10X	Glu	Stop	LB1	1	Htz	Wa		?	Flemish - Walloon	28
248	2	91	10	GAG	TAG	G->T	No	Spe I +	E10X	Glu	Stop	LB1	1	Htz	Wa		? - F 6/30	Spanish	13
335	2	91	10	GAG	TAG	G->T	No	Spe I +	E10X - MOROCCO	Glu	Stop	LB1	1		Wa		?	Jewish Ashkenazi	23
215	2	131	23	TGG	TAG	G->A	No	BsiY I -	W23X	Trp	Stop	LB1	1	Htz	Wa		? - F	Danish	27
271	2	131	23	TGG	TAG	G->A	No	BsiY I -	W23X	Trp	Stop	LB1	1	Htz	Wa		? - F	German	*4
300	2	131	23	TGG	TAG	G->A	No	BsiY II -	W23X	Trp	Stop	LB1	1	Htz	Wa		?	Belgian	**2
318	2	139	26	GAT	AAT	G->A	Yes		D26N - HYOGO	Asp	Asn	LB1		Htz	Wa		P	Japanese	*3
261	2	148	29	GCT	ACT	G->A	Yes	Dra III +	A29T	Ala	Thr	LB1		Htz	Wa		?	Austrian	*2
283	2	148	29	GCT	ACT	G->A	Yes	Dra III +	A29T	Ala	Thr	LB1		Htz	Wa		?	S. Afr. Coloured	**1
324	2	166	35	TCT	CCT	T->C	No	Hae III +	S35P	Ser	Pro	LB1			Wa		F 2/742	Norwegian	21
222	3	232	57	CGT	TGT	C->T	Yes	Hae III -	R57C	Arg	Cys	LB2					P	British	8
242	3	232	57	CGT	TGT	C->T	Yes	Hae III -	R57C	Arg	Cys	LB2					P	S. Afr. Indian	12
282	3	253	64	CAG	TAG	C->T	No		Q64X	Gln	Stop	LB2	1	Htz	Wa		?	S. Afr. Jewish	**3
216	3	259	66	TGG	GGG	T->G	No	BsiY I +	W66G	Trp	Gly	LB2		Htz	Wa		F	Danish	27
277	3	259	66	TGG	GGG	T->G	No	BsiY I +	W66G	Trp	Gly	LB2	3 or 5	Htz	Wa		?	German	*4
286	3	259	66	TGG	GGG	T->G	No	BsiY I +	W66G	Trp	Gly	LB2		Hmz	ab	?	?	Afrikaner	*1
223	3	260	66	TGG	TAG	G->A	No	Xba I +	W66X	Trp	Stop	LB2					P	British	8
224	3	266	68	TGC	TAC	G->A	No	Rsa I +	C68Y	Cys	Tyr	LB2					P	British	8
225	3	268	69	GAT	AAT	G->A	Yes		D69N	Asp	Asn	LB2					P	British	8
263	3	283	74	TGC	GGC	T->G	No		C74G	Cys	Gly	LB2		Htz	Wa		P	Spanish	18
227	3	301	80	GAG	del1a	Stop at 204			301delG	Glu	Fr.	LB2					F 3/791	British	8
262	3	311	83	TGT	TTT	G->T	No		C83F	Cys	Phe	LB2		Htz	Wa		P	British	*1
240	3-4	313	84	CCC	TCC	C->T	No		P84S	Pro	Ser	LB3		Htz	Wa		P	Finnish	11
217	4	335	91	GAC	del10b	Stop at 201			335dal10	Asp	Fr.	LB3		Htz	Wa		P	Danish	9
355	4	337	92	GAG	TAG	G->T	No	Rma I +	E92X	Glu	Stop	LB3	1	Htz	Wa		?	Flemish	28
317	4	344	94	CGC	CAC	G->A	Yes		R94H - FUKUOKA	Arg	His	LB3		Htz	Wa		P	Japanese	*3
301	4	347	95	TGC	TTC	G->T	No	Fnu4H I -	C95F	Cys	Phe	LB3		Htz	Wa		P	Belgian	**2
252	4	429	122	TGC	TGA	C->A	No	Age I +	C122X	Cys	Stop	LB3	1	Htz	Wa		F 13/70 (We.) 3/80 (Fl.)	Flemish - Walloon	15
269	4	460	133	CAG	TAG	C->T	No	Rma I +	Q133X	Gln	Stop	LB4		Htz	Wa		P	Spanish	18
325	4	465	134	TGC	TGA	C->A	No		C134X	Cys	Stop	LB4			Wa		F 12/742	Norwegian	21
228	4	479	139	TGC	TAC	G->A	No		C139Y	Cys	Tyr	LB4					P	British	8
299	4	482	140	ATC	ACC	T->C	No		I140T	Ile	Thr	LB4		Htz	Wa		P	Costa Rican	*1
276	-4	500	146	TGC	TAC	G->A	No		C146Y	Cys	Tyr	LB4		Htz	Wa		P	German	*4
229	4	502	147	GAC	AAC	G->A	Yes		D147N	Asp	Asn	LB4					P	British	8
249	4	518	152	TGC	del1b	Stop at 204		Taq I +	518delG	Cys	Fr.	LB4	1	Htz	Wa		F 4/30	Spanish	13
272	4	518	152	TGC	TAC	G->A	No		C152Y	Cys	Tyr	LB4		Htz	Wa		P	German	*4
344	4	519	152	TGC	TGG	C->G	No	BsiY I +	C152W	Cys	Trp	LB4		Htz	Wa		P	German	*5
326	4	523	154	GAT	TAT	G->T	No		D154Y	Asp	Tyr	LB4			Wa		P	Norwegian	21
302	4	527	155	GGC	GTC	G->T	No	BspW I -	G155V	Gly	Val	LB4		Htz	Wa		P	Belgian	**2
264	4	530	156	TCG	TTG	C->T	Yes		S156L	Ser	Leu	LB4		Htz	Wa		R	Spanish	18
345	4	533	157	GAT	ins8b	Stop at 178			533ins8	Asp	Fr.	LB4		Htz	Wa		? - F	German	*5
343	4	554	164	AGG	ins1b	Stop at 178			553insG	Arg	Fr.			Htz	Wa		P	German	*5
338	4	558	165	GGT	del1a	Stop at 204			556delG - ISRAEL	Gly	Fr.				ab	6 5	P	Arab Moslem	23
290	4	558	165	GGT	ins1c	Stop at 178			558insG	Gly	Fr.			Htz	Wa		P	German	19
295	4	617	185	AGT	del1b	Stop at 204			617delG	Ser	Fr.	LB5		Htz	Wa		P	French	**3
230	4	646	195	TGT	del1a	Stop at 204			646delT	Cys	Fr.	LB5					P	British	8
342	4	661	200	GAC	AAC	G->A	Yes		D200N	Asp	Asn	LB5		Htz	Wa		P	German	*5
266	4	662	200	GAC	GGC	A->G	No	Msp I +	D200G	Asp	Gly	LB5	2B	Htz	Wa		?	Spanish	18
273	4	662	200	GAC	GGC	A->G	No	Msp I +	D200G	Asp	Gly	LB5	2B	Htz	Wa		?	German	*4
280	4	662	200	GAC	GGC	A->G	No	Msp I +	D200G	Asp	Gly	LB5		Htz	Wa		? - F	Afrikaner	*1
243	4	661	200	GAC	TAC	G->T	No		D200Y	Asp	Tyr	LB5		Htz	Wa		?	S. Afr. Indian	12

A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T
265	4	661	200	GAC	TAC	G->T	No		D200Y	Asp	Tyr	LB5		Htz	Wa		?	Spanish	18
285	4	671	203	GAC	GCC	A->C	No	Hae III +	D203A	Asp	Ala	LB5		Htz	Wa		P	S. Afr. Coloured	**1
250	4	681	206	GAC	ins7c	Stop at 216			681ins7	Asp	Fr.	LB5	1	Htz	Wa		P	French-Canadian	14
239	4	681	206	GAC	ins8c	Ins			681ins8	Asp	Fr.	LB5		Htz	Wa		P	Costa Rican	10
245	4	682	207	GAG	AAG	G->A	Yes		E207K	Glu	Lys	LB5	2B	Htz	Wa		? - F 2/7	S. Afr. Indian	12
336	4	682	207	GAG	CAG	G->C	No	EcoR II +	E207Q - IRAQ	Glu	Gln	LB5	2B		Wa		?	Jewish Ashkenazi	23
354	5	731	223	TCT	TAT	C->A	No		S223Y	Ser	Tyr	LB6		Htz	Wa		P	Flemish	28
231	5	736	225	GGA	del1a	Stop at 263			736delG	Gly	Fr.	LB6					P	British	8
255	5	757	232	CGG	TGG	C->T	Yes	Msp I -	R232W	Arg	Trp	LB6		Htz	ab	2140+5: G>A	P	Austrian	*2
358	5	767	235	GAC	GAG	A->G	No		D235E	Asp	Glu	LB6		Htz	Wa		?	Finnish	25
256	6	828	255	TGC	TGA	C->A	No		C255X	Cys	Stop	LB7		Htz	Wa		F 2/508	Austrian	*2
353	6	829	256	GAG	AAG	G->A	Yes		E256K	Glu	Lys	LB7		Htz	Wa		?	Walloon	28
303	6	855	264	CAC	CAA	C->A	No		H264Q	His	Gln	LB7		Htz	Wa		P	Belgian	**2
341	6	862	267	GAA	AAA	G->A	Yes		E267K	Glu	Lys	LB7		Htz	Wa		P	German	*5
253	6	902	280	GAC	GGC	A->G	No		D280G	Asp	Gly	LB7		Htz			F 5/115	Greek	16
294	6	910	283	GAC	AAC	G->A	No	BstK I -	D283N	Asp	Asn	LB7	2B		Wa		?	Irish	**3
278	6	917	285	TCA	TTA	C->T	No		S285L	Ser	Leu	LB7	2B	Hmz	aa		?	German	29
279	6	917	285	TCA	TTA	C->T	No		S285L	Ser	Leu	LB7	2B	Htz	Wa		? - F	Afrikaner	*1
232	6	931	290	AAA	del2a	Stop at 298			931delAA	Lys	Fr.	LB7					P	British	8
233	6	938	292	TGC	TAC	G->A	No	Rsa I +	C292Y	Cys	Tyr	LB7					F 4/791	British	8
234	7	979	306	CAC	TAC	C->T	No		H306Y	His	Tyr	EGFA					P	British	8
331	7	985	308	TGC	GGC	T->G	No		C308G - POLAND	Cys	Gly	EGFA			Wa		P	Jewish Ashkenazi	23
318	7	1012	317	TGC	CGC	T->C	No	Hae II +	C317R - GIFU	Cys	Arg	EGFA		Htz	Wa		P	Japanese	*3
235	7	1024	321	GAC	AAC	G->A	Yes		D321N	Asp	Asn	EGFA					P	British	8
304	7	1027	322	GGC	AGC	G->A	Yes	Alu I -	G322S	Gly	Ser	EGFA	2Bor5	Htz	Wa		?	Belgian	**2
337	7	1027	322	GGC	AGC	G->A	Yes	Alu I -	G322S - SYRIA	Gly	Ser	EGFA	2Bor5		Wa		?	Jewish Ashkenazi	23
236	7	1033	324	CAG	TAG	C->T	No	Pvu II -	Q324X	Gln	Stop	EGFA					P	British	8
333	7	1046	328	CAG	del1b	Stop at 368			1046delA - RUSSIA-HUNGARY	Gln	Fr.	EGFA			Wa		P	Jewish Ashkenazi	23
254	7	1048	329	CGA	TGA	C->T	Yes		R329X	Arg	Stop	EGFA					? - F 9/78	British	17
292	7	1048	329	CGA	TGA	C->T	Yes		R329X	Arg	Stop	EGFA		Htz	Wa		? - F	German	19
305	7	1058	331	TGC	TGG	C->G	No		C331W	Cys	Trp	EGFA		Htz	Wa		P	Belgian	**2
319	8	1066	335	GAT	CAT	G->C	No	Taq I -	D335H - KANAGAWA	Asp	His	EGFB		Htz	Wa		P	Japanese	*3
308	8	1066	335	GAT	TAT	G->T	No	Taq I -	D335Y	Asp	Tyr	EGFB		Htz	Wa		?	Belgian	**2
327	-8	1066	335	GAT	TAT	G->T	No	Taq I -	D335Y	Asp	Tyr	EGFB			ab	?	? - F 8/742	Norwegian	21
251	8	1124	354	TAC	TGC	A->G	No	Fnu4H I +	Y354C	Tyr	Cys	EGFB			Wa		F 2/88	French-Canadian	14
298	8	1133	357	CAG	CCG	A->C	No	Bsp1286 I +	Q357P	Gln	Pro	EGFB		Htz	Wa		P	British	**3
284	8	1154	364	CTG	CGG	T->G	No	Alu I -	L364R	Leu	Arg	EGFB		Htz	Wa		P	S. Afr. Coloured	**1
214	8	1176	371	TGC	TGA	C->A	No		C371X	Cys	Stop	EGFB	1	Htz	Wa		P	S. Afr. Indian	26
328	9	1201	380	CTC	GTC	C->G	No	Mae II +	L380V	Leu	Val	EGF			Wa		F 5/742	Norwegian	21
320	9	1207	382	TTC	CTC	T->C	No		F382L - WAKAYAMA	Phe	Leu	EGF		Htz	Wa		P	Japanese	*3
218	9	1210	383	ACC	CCC	A->C	No		T383P	Thr	Pro	EGF		Htz	Wa		P	Danish	27
246	9	1215	384	AAC	AAG	C->G	No	Msp I -	N384K	Asn	Lys	EGF		Htz	Wa		P	S. Afr. Indian	12
314	9	1217	385	CGG	CAG	G->A	Yes	Msp I -	R385Q	Arg	Gln	EGF		Htz	Wa		P	S. Afr. Black - Sotho	**2
332	9	1216	385	CGG	TGG	C->T	Yes	Msp I -	R385W - RUMANIA	Arg	Trp	EGF			Wa		P	Jewish Ashkenazi	23
307	9	1243	394	GAC	CAC	G->C	No	Fnu4H I +	D394H	Asp	His	EGF		Htz	Wa		P	Belgian	**2
291	9	1247	395	CGG	CAG	G->A	Yes	Msp I -	R395Q	Arg	Gln	EGF		Htz	Wa		?	German	19
339	9	1247	395	CGG	CAG	G->A	Yes	Msp I -	R395Q	Arg	Gln	EGF		Htz	Wa		?	German	*6
237	9	1246	395	CGG	TGG	C->T	Yes	Msp I -	R395W	Arg	Trp	EGF					? - F4/791	British	8
329	9	1248	395	CGG	TGG	C->T	Yes	Msp I -	R395W	Arg	Trp	EGF			Wa		? - F 9/742	Norwegian	21
309	9	1257	398	TAC	TAG	C->G	No	Rsa I -	Y398X	Tyr	Stop	EGF		Htz	Wa		P	Belgian	**2
288	9	1284	407	AAC	AAG	C->G	No	Mae II -	N407K	Asn	Lys	EGF		Hmz	ab	6	P	S. Afr. Jewish	**3
212	9	1285	408	GTG	ATG	G->A	Yes	Mae II -	V408M	Val	Met	EGF	5	Hmz	aa		F	Greek	30

Table 1. *continued*

A	B	C	D	E	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T
321	9	1285	408	GTG	ATG	G->A	Yes	Mae II -	V408M - OSAKA	Val	Met	EGF	5	Htz	Wa		?	Japanese	*3
347	9	1294	411	CTG	GTG	C->G	No		L411V	Leu	Val	EGF		Htz	Wa		P	German	*5
267	9	1301	413	ACG	AAG	C->A	No		T413K	Thr	Lys	EGF	5	Htz	Wa		?	Spanish	18
309	9	1301	413	ACG	AGG	C->G	No		T413R	Thr	Arg	EGF		Htz	Wa		?-F	Belgian	**2
352	9	1301	413	ACG	AGG	C->G	No		T413R	Thr	Arg	EGF		Htz	Wa		?-F	Flemish - Walloon	28
330	9	1307	415	GTG	GCG	T->C	No	Msc I -	V415A	Val	Ala	EGF			Wa		P	Dutch	22
298	9	1329	422	TGG	TGC	G->C	No		W422C	Trp	Cys	EGF	2Bor5	Htz	Wa		?	S. Afr. English - British	**3
258	10	1372	437	AGA	del2a	Stop at 438			1372delAG	Arg	Fr.	EGF		Htz	Wa		P	Austrian	*2
323	10	1469	469	TGG	TAG	G->A	No	Rma I +	W469X	Trp	Stop	EGF	1	Hmz	aa		P	German	29
257	10	1474	471	GAC	AAC	G->A	Yes	Xcm I +	D471N	Asp	Asn	EGF		Htz	We		F 2/494	Austrian	*2
334	10	1567	502	GTG	del9a	del		Bam HI -	1567del9 - IRAQ	Val	Fr.	EGF			Wa		P	Jewish Ashkenazi	23
289	11	1646	528	GGT	GAT	G->A	No		G528D	Gly	Asp	EGF	2A	Htz	We		?	Greek	**3
219	11	1650	529	GTG	del1c	Stop at 546			1650delG	Val	Fr.	EGF		Htz	Wa		P	Danish	27
220	12	1730	556	TGG	TCG	G->C	No		W556S	Trp	Ser	EGF	1	Htz	Wa		F 8/61	Danish	9
351	12	1775	571	GGG	GAG	G->A	No		G571E	Gly	Glu	EGF	5	Htz	Wa		?	Flemish - Walloon	28
322	12	1784	574	CGG	CAG	G->A	Yes	Msp I -	R574Q	Arg	Gln	EGF		Htz	Wa		P	Japanese	*3
310	12	1823	587	CCC	CTC	C->T	No		P587L	Pro	Leu	EGF		Htz	We		P	Belgian	**2
311	12	1840	593	TTT	del2a	Stop at 600			1840delTT	Phe	Fr.	EGF		Htz	Wa		P	Belgian	**2
350	13	1864	601	GAT	TAT	G->T	No	EcoR V -	D601Y	Asp	Tyr	EGF		Htz	Wa		P	Flemish	28
349	13	1978	639	CAG	TAG	C->T	No	Rma I +	Q639X	Gln	Stop	EGF		Htz	Wa		P	Flemish	28
259	14	1998	645	TGG	TGA	G->A	No		W645X	Trp	Stop	EGFC		Htz	Wa		P	Austrian	*2
268	14	2000	646	TGT	TAT	G->A	No		C646Y	Cys	Tyr	EGFC	2A	Htz	We		R	Spanish	18
346	14	2001	646	TGT	TGA	T->A	No		C646X	Cys	Stop	EGFC			Wa		P	Swedish	31
357	14	2054	664	CCG	CTG	C->T	Yes	Pst I +	P664L	Pro	Leu	EGFC					?-F 7/915	Dutch	24
312	14	2054	664	CCG	CTG	C->T	Yes	Pst I +	P664L	Pro	Leu	EGFC	2B	Htz	Wa		?-F	Belgian	**2
348	14	2054	664	CCG	CTG	C->T	Yes	Pst I +	P664L	Pro	Leu	EGFC	2B	Htz	Wa		?-F	Flemish - Walloon	28
315	14	2056	665	CAG	TAG	C->T	No	Acl I -	Q665X	Gln	Stop	EGFC		Htz	Wa		P	Costa Rican	20
270	14	2085	674	ACC	del19c	Stop at 701			2085del19	Thr	Fr.	EGFC		Htz	Wa		P	Spanish	18
293	14	2092	677	TGC	del1a	Stop at 707			2092delT	Cys	Fr.	EGFC		Htz	Wa		?	Greek-French	**3
260	14	2093	677	TGC	TAC	G->A	No		C677Y	Cys	Tyr	EGFC		Htz	Wa		F 3/530	Austrian	*2
313	14	2096	678	CCG	CTG	C->T	Yes	Msp I -	P678L	Pro	Leu	EGFC		Htz	We		?-F	Belgian	**2
221	15	2177	705	ACC	ATC	C->T	No		T705I	Thr	Ile	OLS		Htz	Wa		?	Danish	27
244	16	2356	765	AGC	TGC	A->T	No		S765C	Ser	Cys	OLS		Hmz	aa		P	S. Afr. Indian	12
281	16-17	2389	776	GTG	ATG	G->A	Yes	Nla III +	V776M	Val	Met	TM		Htz	Wa		?	Afrikaner	*1
340	17	2392	777	CTC	del9a	del			2392del9	Leu	Fr.	TM		Htz	ab	186	P	German	*6
287	17	2441	793	CGG	CAG	G->A	Yes	Alu I +	R793Q	Arg	Gln	CP		Htz	Wa		P	S. Afr. Black - Xhosa	**2

Each line represents a single LDLR mutation report. The columns contain the following informations and abbreviations:

A: Report number.

B: Exon number in which the mutation occured. Exons are numbered according to Südhof *et al.* (6) with respect to the translational initiation site given by Yamamoto *et al.* (5).

C: Nucleotide position in which the mutation occured.

D: Codon number in which the mutation occured. Codons are numbered according to Yamamoto *et al.* (5). Therefore, the 21 amino acids of the signal peptide (exon 1) are numbered in negative (from –21 to –1). Codon number 1 is the last codon of exon 1 and encodes the first amino acid (Ala) of the mature LDL receptor. If the mutation spans more than one codon, e.g., there is a deletion of several bases, only the first (5′) deleted codon is entered.

E: Normal base sequence of the codon in which the mutation occured.

F: Mutated base sequence of the codon in which the mutation occured. If the mutation is a base pair deletion or insertion, this is indicated by «del» or «ins» followed by the number of bases deleted or inserted and the position of this deletion or insertion in the codon (a, b or c). The nucleotide position is the first that is deleted or the one preceding the insertion. For example, «del19c» is a deletion of 19 bases including the third base of the codon, «ins8b» is an insertion of 8 bases occuring between the second and the third base of the codon.

G: Concerns base substitutions. It gives the base change, by convention, read from the coding strand. If the mutation predicts a premature protein-termination, the novel stop codon position is given, e.g., «stop at 204».

H: Concerns events occuring at a CpG dinucleotide (only C→T or G→A).

I: Concerns the restriction site that is lost, e.g., «Msp I –», or created, e.g., «Taq I +», by the mutation.

J: Mutation name according to Beaudet *et al.* (32). Missense mutations are designated by the codon number flanked by the single letter code of the normal amino acid prior and of the mutant amino acid after (e.g., Val to Met at codon 408 is designated «V408M»). Nonsense mutations are designated similarly exept that X is used to indicate any termination codon (e.g., Cys to stop at codon 134 is designated «C134X»). Frameshift, insertion and deletion mutations are designated by the nucleotide number followed by «ins» for insertion or «del» for deletion. The nucleotide position is the first that is deleted or the one preceding it in the case of insertions. Exact nucleotides are indicated for two or less bases (e.g., 617delG). For three or more bases, the insertion or deletion is specified by the size of the change (e.g. 681ins8 indicates a 8 bp insertion starting after nucleotide 681). For many of the mutations that have been reported this nomenclature has not been used. Therefore, the original name also appears in this column. These names were given according to the population or the city in which the mutation was reported first (e.g. TOKYO).

K: Wild type amino acid.

L: Mutant amino acid. Deletion and insertion mutations which result in a frameshift are designated by «Fr. »; Nonsense mutations are designated by «Stop».

M: Protein domain in which the mutation occurs. «SP» for the signal peptide, «LB» for the ligand binding domain, «EGF» for the Epidermal Growth Factor precursor like domain, «OLS» for the O-linked sugar chains domain, «TM» for the transmembrane domain, and «CP» for the cytoplasmic domain. In the ligand-binding domain (LB), each of the seven repeats are numbered separately and according to their position with respect to the N-terminal end of the protein.

N: Functional class as defined by Hobbs *et al.* (40).

O: Clinical status according to Goldstein *et al.* (2): «Hmz» indicates homozygotes and «Htz» indicates heterozygotes.

P: Genotype: «aa» indicates homozygotes, «ab» indicates compound heterozygotes, and «Wa» indicates heterozygotes. Empty cases appear when no information is available.

Q: Number of the report in which the second mutation identified in a compound heterozygote is described. When the second mutation is one of those omitted in the database, this mutation is briefly described with respect to the coding sequence. Finally, «?» indicates that the second mutation has not been identified.

R: Recurence of the mutation. «F» indicates a founder effect, «F 2/140» indicates that the mutation was found in two unrelated probands in a sample 140 FH patients, «R» indicates recurrent mutations, «?» indicates mutations that have been identified in at least two unrelated probands of different ethnic backgrounds but for which LDLR gene haplotypes are not described, «?–F» indicates mutations for which LDLR gene haplotypes are not described (or incomplete) and that either are associated with a founder effect in the proband's ethnic or geographic origin, or have been identified in at least two unrelated probands of the same ethnic or geographic background, and «P» indicates mutations identified, to date, in a single proband.

S: Ethnic or geographic background of the proband.

T: Reference number indicating the publication in which the mutation is described. Full citations (authors, year, title, journal, volume, pages) are provided with the database. If the same mutation has been reported for the same patient in different papers, only one entry is made.

*Indicates the co-authors who provided the information: *1 (Rochelle Thiart and Maritha J. Kotze), *2 (Helena Schmidt and Gert M. Kostner), *3 (Yasuko Miyake and Taku Yamamura), *4 (Heike Baron and Herbert Schuster), *5 (Margit Ebhardt and Manfred Stuhrmann) and *6 (Hartmut Schmidt).

**Indicates submitted papers: **1 (O.Loubser *et al.*), **2 (A.Peeters *et al.*) and **3 (M.Callis *et al.*).

Table 2. Each line represents a single LDLR mutation report

First mutation											Second mutation											O	R	S	T
J	B	C	D	E	F	G	K	L	M	P	J	B	C	D	E	F	G	K	L	M	P				
W-18X	1	12	-18	TGG	TGA	G->A	Try	Stop	SP	Wa	E256K	6	829	256	GAG	AAG	G->A	Glu	Lys	LB7	Wa	Htz	P	Spanish	18
Q71E	3	274	71	CAA	GAA	C->G	Gln	Glu	LB2	Wa	313+1(G->C)	3	313+1	-	-	-	G->C	-	-	LB3	Wa	Htz	P	Spanish	18
C95R	4	346	95	TGC	CGC	T->C	Cys	Arg	LB3	Wa	D679E	14	2100	679	GAC	GAG	C->G	Asp	Glu	EGFC	Wa	Htz	P	Spanish	18
854ins6	4	654	197	GGT	Ins6c	Ins	Gly	Fr	LB5		657del5	4	657	198	GGC	del5c	del	Gly	Fr	LB5			P	German	37
C281Y	6	905	281	TGC	TAC	G->A	Cys	Tyr	LB7	Wa	1706-10(G->A)	11	1706-10	-	-	-	G->A	-	-	EGF	Wa	Htz	P	Spanish	18
D333A	8	1061	333	GAT	GTC	A->C	Asp	Ala	EGFB	aa	2140+5(G->A)	14	2140+5	-	-	-	G->A	-	-	EGF	aa	Hmz	P	Austrian	*2
1115del9	8	1115	351	GAG	del9b	del	Glu	Fr	EGFB	Wa	1151ns6	8	1115	351	GAG	Ins6a	Ins	Glu	Fr	EGFB	Wa	Htz	F 2/-	Japanese	34
Q363X	8	1150	363	CAG	TAG	C->T	Gln	Stop	EGFB	Wa	D365E	8	1158	365	GAC	GAG	C->G	Asp	Glu	EGFB	Wa	Htz	P	Cypriot	33
N543H	11	1690	543	AAT	CAT	A->C	Asn	His	EGF	Wa	2393del9	17	2393	777	CTC	del9b	del	Leu	Fr	TM	Wa	Htz	? - F 2/63	Danish	36
N543H	11	1690	543	AAT	CAT	A->C	Asn	His	EGF	Wa	2393del9	17	2393	777	CTC	del9b	del	Leu	Fr	TM	Wa	Htz	? - F 10/184	Dutch	35
A585T	12	1816	585	GCC	ACC	G->A	Ala	Thr	EGF	Wa	G654S	14	2023	654	GGC	AGC	G->A	Gly	Ser	EGFC	Wa	Htz	F 2/530	Austrian	*2

Footnotes as for Table 1.

current version as well as specific phenotypic data would be gratefully received by the corresponding authors.

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APPENDIX 3

A third major locus for autosomal dominant hypercholesterolemia maps to 1p34.1-p32

Varret M, Rabés JP, Saint-Jore B, Cenarro A, Marinoni JC, Civeira F, Devillers M, Krempf M, Coulon M, Thiart R, Kotze MJ, Schmidt H, Buzzi JC, Kostner GM, Bertolini S, Pocovi M, Rosa A, Farnier M, Martinez M, Junien C, Boileau C

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A Third Major Locus for Autosomal Dominant Hypercholesterolemia Maps to 1p34.1-p32

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Summary

Autosomal dominant hypercholesterolemia (ADH), one of the most frequent hereditary disorders, is characterized by an isolated elevation of LDL particles that leads to premature mortality from cardiovascular complications. It is generally assumed that mutations in the *LDLR* and *APOB* genes account for ADH. We identified one large French pedigree (HC2) and 12 additional white families with ADH in which we excluded linkage to the *LDLR* and *APOB*, implicating a new locus we named "*FH3*." A LOD score of 3.13 at a recombination fraction of 0 was obtained at markers *D1S2892* and *D1S2722*. We localized the *FH3* locus to a 9-cM interval at 1p34.1–p32. We tested four regional markers in another set of 12 ADH families. Positive LOD scores were obtained in three pedigrees, whereas linkage was excluded in the others. Heterogeneity tests indicated linkage to *FH3* in ~27% of these non-*LDLR*/non-*APOB* ADH families and implied a fourth locus. Radiation hybrid mapping located four candidate genes at 1p34.1-p32, outside the critical region, showing no identity with *FH3*. Our results show that ADH is genetically more heterogeneous than conventionally accepted.

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Introduction

Autosomal dominant hypercholesterolemia (ADH) is an inherited disorder of lipid metabolism, characterized by a selective increase of LDL particles in plasma (type IIa hyperlipoproteinemia) giving rise to tendon and skin xanthomas, arcus corneae, and premature mortality from cardiovascular complications (Goldstein and Brown 1989). Twenty-five years ago, Goldstein and Brown (1974) showed that ADH results from defects in the cell-surface receptor that removes LDL particles from plasma. They coined the disorder "familial hypercholesterolemia" (FH; MIM 143890). The LDL-particle receptor is a ubiquitous transmembrane glycoprotein of 839 amino acids that mediates the transport of LDL particles into cells via endocytosis (Goldstein and Brown 1974). The LDL-particle receptor gene (*LDLR*) was cloned by Yamamoto et al. (1984) and mapped to 19p13.1-13.3 by Lindgren (1985), and >600 mutations scattered throughout the 45-kb gene have now been reported (Varret et al. 1997*a*, 1997*b*, 1998; LDLR Mutation Database). Innerarity et al. (1987) demonstrated the genetic heterogeneity of ADH by reporting hypercholesterolemic patients with normal LDL-particle receptor activity and defective apolipoprotein B-100 that displayed low affinity for its receptor. This new molecular disorder was called "familial ligand-defective apolipoprotein B-100" (FDB; MIM 144010). The *APOB* gene spans 43 kb, is divided into 29 exons, and maps to chromosome 2p23-p24 (Law et al. 1985). To date, three mutations in the *APOB* gene associated with ADH have been reported: the R3500Q mutation is the most frequent, whereas the R3531C and the R3500W are relatively rare (Rabès et al. 1997). Thus, it convention-

ally is assumed that only two distinct genetic disorders account for the ADH phenotype.

The existence of a greater level of genetic heterogeneity has never been formulated clearly. However, careful examination of the literature shows that, by different approaches, a few families have been identified in which the ADH phenotype was not associated with defects in the *LDLR* and *APOB* genes. Lestavel-Delattre et al. (1994) studied 41 patients with the ADH phenotype (plasma LDL-cholesterol level >95th percentile, xanthomas, and/or personal or family history of coronary artery disease) and found normal LDL-particle receptor activity and normal LDL-particle binding in nine patients who did not carry the R3500Q mutation in the *APOB* gene. Further studies showed that six of these patients carried a mutation in the *LDLR* gene, but for the three other ADH subjects the genetic origin of the disease is presently unknown (Nissen et al. 1998). These results could be explained by a defect in the regulation of the expression of the *LDLR* gene or by the involvement of another locus. This latter hypothesis was indirectly confirmed by other teams (Miserez and Keller 1995; Sun et al. 1997) who reported ADH families in which they found normal LDL-particle receptor activity and a genetic exclusion of the *LDLR* and *APOB* genes. Furthermore, several cases of pseudohomozygous type IIa hypercholesterolemia have been reported by Masana et al. (1990), who showed that probands of this rare autosomal recessive hypercholesterolemia (ARH) have normal levels of LDL particles and normal LDL-particle receptor activity. In the present study we found 13 families that not only support these observations but also give evidence for the existence of a third gene involved in ADH, which we named "*FH3.*" To identify *FH3,* we performed linkage analyses and exclusion mapping in a large French ADH family in which defects in *LDLR* or *APOB* have been conclusively ruled out. After positive LOD scores were obtained, confirmation of linkage in this first stage was sought in a second sample of 12 ADH families originating from France, Austria, Spain, Belgium, and New Zealand and presenting the typical ADH phenotype. Two Sardinian ARH families were also tested.

Families and Methods

Families and Clinical Evaluation

Family HC2 is a large ADH pedigree comprising three generations (fig. 1). All affected members were characterized by levels of total cholesterol above the 97.5th percentile when compared with other French indivuduals matched by age and sex (Steinmetz 1990). The proband (II-9) is a 36-year-old woman, ascertained at age 17 years with 3.32 g/l total cholesterol, 2.36 g/l LDL cholesterol, 0.48 g/l HDL cholesterol, 0.61 g/l triglycerides, and arcus corneae. Her sister (II-7) is a 40-year-old woman ascertained at age 20 years with 4.10 g/l total cholesterol, 3.12 g/l LDL cholesterol, 0.52 g/l HDL cholesterol, 0.75 g/l triglycerides, and arcus corneae, tendon xanthomas, and xanthelasmas. Distribution of total and LDL cholesterol values in all tested HC2 members is bimodal, indicating the autosomal dominant transmission of the disease (data not shown). All lipid values were confirmed with at least a second lipid measurement. Among the 39 family members, seven (II-3, III-3, III-4, III-5, III-6, II-13, and III-15) were scored as "unknown" in linkage analyses because they presented with borderline lipid values or because only one lipid determination was available. In the second set of families, all affected subjects were scored according to their level of total or LDL cholesterol above the 95th percentile when compared with other individuals from their origin population matched by age and sex (Civeira et al. 1990; Moreda et al. 1990; Williams et al. 1993). Affected subjects ($n = 46$) had mean lipid values as follows: 3.02 ± 0.81 g/l total cholesterol, 2.25 ± 0.76 g/l LDL cholesterol, 0.52 ± 0.19 g/l HDL cholesterol, and 1.23 ± 0.51 g/l triglycerides. Unaffected subjects ($n = 54$) had mean lipid values as follows: 2.04 ± 0.27 g/l total cholesterol, 1.32 ± 0.27 g/l LDL cholesterol, 0.52 ± 0.12 g/l HDL cholesterol, and 0.88 ± 0.36 g/l triglycerides. Subjects with an unknown phenotypic status ($n = 6$) had the following mean lipid values: 2.86 ± 0.33 g/l total cholesterol, 1.94 ± 0.27 g/l LDL cholesterol, 0.52 ± 0.10 g/l HDL cholesterol, and 1.46 ± 0.83 g/l triglycerides. All these values were confirmed with at least a second lipid measurement. Finally, in the Sardinian ARH families, the four affected subjects presented an elevated level of total (>4 g/l) and LDL (>3.5 g/l) cholesterol, and normal triglyceride (<1.5 g/l) and HDL (<0.5 g/l) levels. All subjects gave informed consent.

DNA Analysis and PCR Amplifications

DNA was isolated from whole blood samples with use of a method described by Collod et al. (1994). PCR amplifications and electrophoresis were performed under conditions reported by Collod et al. (1994) or adapted from Reed et al. (1994) by use of fluorescent-labeled primers. Alleles observed in the pedigrees were numbered arbitrarily for each marker. All marker-typing data were collected blindly and independently by two investigators (M.V. and J.P.R.), and all linked 1p markers were typed twice. Nonpaternity/nonmaternity was ruled out indirectly because no incompatible phase was found with the numerous highly polymorphic markers tested.

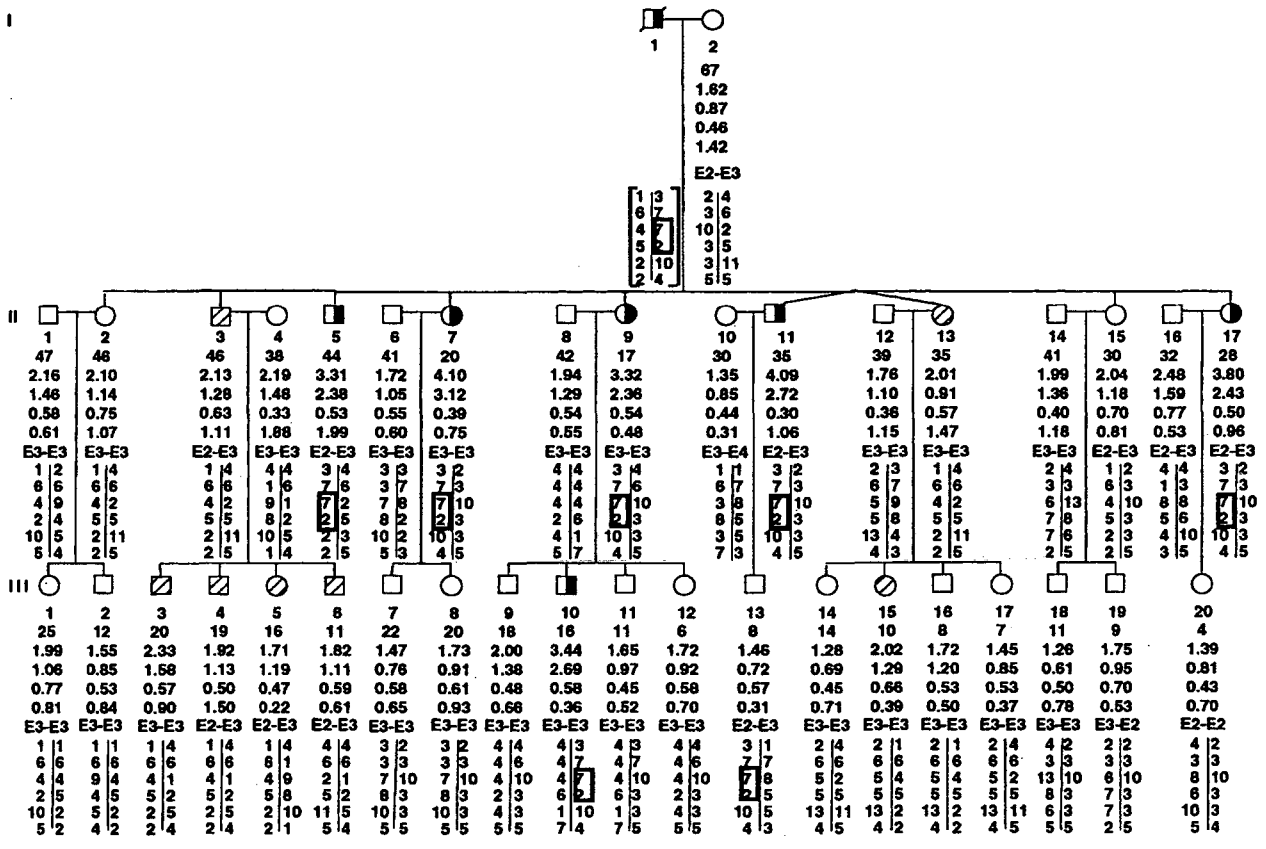

Figure 1 Segregation of chromosome 1p34.1-p32 markers in the French family HC2. Lipid values given under each family-member symbol are (*top to bottom*): age at lipid measurement; levels of total, LDL, and HDL cholesterol (in grams per liter); levels of triglycerides (in grams per liter); and *apo*E genotype (INNO-LiPA *Apo*E, Innogenetics, Murex, Châtillon) (Harrington et al. 1994). Haplotypes (*top to bottom*) at tel-*D1S255-D1S472-D1S2892-D1S2722-D1S211-D1S197*-cen markers are shown for each family member tested. Half-blackened symbols indicate affected members, unblackened symbols indicate unaffected members, and hatched symbols indicate members with an unknown phenotypic status. The haplotype in brackets of subject I-1 was unequivocally deduced. The common region segregating with the disease phenotype in the family is boxed.

Parametric Linkage Analysis

Pairwise and multipoint linkage analyses were performed with SLINK, MLINK, and LINKMAP of the LINKAGE package (Ott 1991) and the VITESSE algorithm (O'Connell and Weeks 1995), with the assumption that ADH is an autosomal dominant disease with a gene frequency of .002. Marker-allele frequencies were calculated with the data from the unrelated family members. Linkage was investigated with the assumption of equal female-to-male recombination rates. The distances between markers used in the LINKMAP analyses were estimated from family data. We assumed the penetrance for disease carriers to be .9, knowing that complete penetrance can lead to false exclusion and that reduced penetrance has been reported in FH and FDB. Penetrance for noncarriers was assumed to be zero. EXCLUDE (Edwards 1987) was used to produce the exclusion map. This program estimates the positional likelihood of the disease locus on each chromosome and the percentage of probability of the disease locus to be on any of the 22 autosomes. Heterogeneity tests were performed with HOMOG (Ott 1983), which computes likelihoods of linkage under genetic heterogeneity by estimating recombination fractions (θ) and the proportion of families showing linkage to the markers under study (α).

Nonparametric Linkage Analysis

GENEHUNTER (Kruglyak et al. 1996) performs complete multipoint analysis to infer the degree of identity-by-descent sharing among all affected family members at each map point. We used the modified version of this program, GENEHUNTER-PLUS (Kong and Cox 1997), which has been shown to be less conservative, particularly when data are less than perfectly informative. GENEHUNTER-PLUS calculates a semiparametric LOD score (ghpLS) by using a single parameter that is a measure of the inheritance vector in the pedigree and allele sharing.

Table 1

Description of the *FH3* Families Studied

Family	Origin	No. of Subjects with Status			LDL Receptor Activity[a]	LOD Score at		Mutation Screening in		Reference Population
		Affected	Unaffected	Unknown		LDLR	APOB[a]	LDLR[a]	APOB[b]	
HC2	France	6	25	7	Normal	−6.38	− 4.47	nd	Negative	Steinmetz et al. 1990
HC67	France	4	2	0	nd	−2.75	.56	nd	Negative	Steinmetz et al. 1990
A1	Austria	3	5	3	nd	−1.88	.19	Negative[c]	Negative	Williams et al. 1993
S150	Spain	3	3	0	nd	−1.18	−3.79	nd	Negative	Moreda et al. 1990; Civeira et al. 1990
S108	Spain	3	4	0	nd	−3.53	.82	nd	Negative	Moreda et al. 1990; Civeira et al. 1990
S113	Spain	4	4	0	nd	−1.98	−3.26	nd	Negative	Moreda et al. 1990; Civeira et al. 1990
S517	Spain	4	1	0	nd	−.19	−1.73	Negative	Negative	Moreda et al. 1990; Civeira et al. 1990
S509	Spain	4	2	0	nd	−1.72	−1.72	nd	Negative	Moreda et al. 1990; Civeira et al. 1990
S601	Spain	5	4	0	nd	−2.41	.30	nd	Negative	Moreda et al. 1990; Civeira et al. 1990
S129	Spain	4	0	0	nd	−1.50	−1.61	nd	Negative	Moreda et al. 1990; Civeira et al. 1990.
S206	Spain	4	9	1	nd	−4.53	−2.74	nd	Negative	Moreda et al. 1990; Civeira et al. 1990
B1	Belgium	6	7	0	nd	−4.67	−.88	nd	Negative	Williams et al. 1993
NZ1	N. Zealand	2	3	2	Normal	−.25	−.66	Negative	Negative	Williams et al. 1993
I3	Sardinia	2	5	1	nd	$-\infty$	nd	nd	Negative	Williams et al. 1993
I4	Sardinia	2	10	4	nd	$-\infty$	nd	nd	Negative	Williams et al. 1993

[a] nd = not determined.
[b] R3500Q and R3531C.
[c] Promoter sequence not investigated.

Candidate Gene Mapping

We used the Radiation Hybrid Mapping Panel GENEBRIDGE 4 and performed PCR according to the protocol described by the supplier (Whitehead Institute/MIT Center for Genome Research [WI/MIT]). The primers used to test the candidate genes were reported for SCP2 (He et al. 1991), EPS15 (Genome Database; STS SHGC-14865), and FABP3 (Phelan et al. 1996). Original primers designed in the 3′ region of the *APOER2* gene were forward, 5′-TTCTTGGCTTTGGCGAAGGTC-3′ and reverse, 5′-TTGGGCTGATCTGGAAACGTC-3′.

Results

We have identified a large French family (HC2) in which linkage to either *LDLR* or *APOB* was conclusively excluded. In this family, 22 meioses are investigated. Linkage analysis with *LDLR* gene markers identified two recombinants (one affected subject and one unaffected subject). Linkage to *APOB* was excluded because seven recombinants were identified (two affected subjects and five unaffected subjects). This result was confirmed by a functional test that showed normal binding, internalization, and degradation of control LDL particles in fibroblasts from the proband (data not shown). Furthermore, no family member carried either of the two most frequent hypercholesterolemic *APOB* gene mutations (R3500Q, R3531C; table 1). Finally, to exclude the possibility of type IIb, III, or IV hyperlipoproteinemia in this family, lipid measurements were repeated and apolipoprotein E variants were determined (fig. 1).

We estimated the power of the family for linkage using SLINK (Ott 1991) assuming an autosomal dominant trait. We found a maximum LOD score (Z_{max}) of 4.13 (expected average Z_{max} in 500 replicates was 2.16, with 34% > 3.00) in family HC2, showing that this family was sufficiently informative for linkage. Two hundred four genetic markers from 22 autosomes were tested for linkage to the *FH3* locus. These $(CA)_n$ microsatellite markers spanning the human genome were chosen on the basis of two criteria: heterozygosity and spacing of ~15 cM between adjacent markers (Dib et al. 1996). Each locus was tested for linkage to the ADH phenotype by use of MLINK (Ott 1991) and VITESSE (O'Connell and Weeks 1995). The combined genotype data were also analyzed with EXCLUDE (Edwards 1987): a nonoverlapping exclusion zone of ≥2,955 cM, correspond-

ing to 80% of the genome, was established from the cumulative exclusion intervals for each marker. The EXCLUDE analysis also indicated that the most probable position for the *FH3* locus in family HC2 was on chromosome 1, with a probability of 96%. In this region, a LOD score of 2.13 at $\theta = 0$ had been obtained at *D1S255* (table 2). Other microsatellites were tested around *D1S255*, and a Z_{max} of 3.13 ($\theta = 0$) was obtained at *D1S2892* and *D1S2722* in the HC2 family (table 1). Multipoint linkage analyses did not provide higher LOD scores (data not shown). As shown in figure 1, we found a common region (*D1S2892–D1S2722*) that segregated with the ADH phenotype, localizing *FH3* to a 9-cM interval at 1p34.1-p32 (GENATLAS).

To investigate the reproducibility of linkage, other families from different countries–with 12 presenting ADH and 2 presenting ARH–were collected through an international collaborative effort (table 1). Markers *D1S472*, *D1S2892*, *D1S2722*, and *D1S211* were tested in these families. In three families (S113, S150, and S517) originating from northeast Spain, LOD-score values near their Z_{max} were obtained for these markers. Thus, these families present a very high probability of linkage between the disease and the *FH3* locus on chromosome 1 (fig. 2, table 3). In the two Sardinian ARH families, linkage with all the markers tested was excluded (fig. 3). To investigate whether exclusion of linkage of ADH to 1p could be a result of wrong model specification, we performed model-free linkage analyses by using GENEHUNTER-PLUS (Kong and Cox 1997). Positive ghpLS scores were obtained for families HC2, S150, S113, and S601 (data not shown). This analysis confirmed that the *FH3* nonlinked families outlined by our parametric linkage analyses were not excluded because of misspecification of ADH inheritance mode. Finally, to estimate the proportion of families linked to the *FH3* locus on 1p, we performed an admixture test using HOMOG (Ott 1983), in the whole sample (family HC2 and the 12 ADH families). As shown in table 3, the test was statistically significant with markers *D1S472*, *D1S2892*, and *D1S2722*. Furthermore, the estimated proportion of families with linkage to the *FH3* locus was $\alpha = 27\%$ ($P = .005$) at *D1S2892* and $\alpha = 25\%$ ($P = .005$) at *D1S2722*. Multipoint HOMOG analysis gave a Z_{max} of 2.97 ($\alpha = 25\%$, $P = .0025$) between *D1S2892* and *D1S2722* at $\theta = .01$ from *D1S2892*.

The *FH3* gene maps to 1p34.1-p32, which also harbors numerous genes including those that encode a sterol carrier protein (SCP2; Ohba et al. 1994), a fatty acid–binding protein (FABP3; Phelan et al. 1996), an apolipoprotein E receptor (APOER2; Kim et al. 1997), and an epidermal growth factor receptor–pathway substrate (EPS15; Wong et al. 1994). To determine whether these positional and functional candidate genes mapped within the critical interval defined by *D1S472* and *D1S211* should be further investigated, we used the GENEBRIDGE 4 radiation hybrid panel. The results showed that all these genes did not map within the interval, excluding the possibility of identity between the *FH3* gene and one of these regional candidates (data not shown) and suggesting a novel gene for ADH on 1p34.1-p32.

Discussion

We located a third gene involved in ADH on chromosome 1p34.2-p32 and identified a large French family in which the involvement of either *LDLR* or *APOB* was excluded conclusively. This result was confirmed by a functional test that showed that fibroblasts from the proband showed normal binding, internalization, and degradation of control LDL particles. Furthermore, no family member carried either of the two most frequent hypercholesterolemic *APOB* gene mutations (R3500Q, R3531C). To exclude the possibility of type IIb, III, or IV hyperlipoproteinemia in this family, lipid measurements were repeated and apolipoprotein E variants were determined. Furthermore, linkage was also clearly excluded between the disease gene and the *LPL* locus on

Table 2

Pairwise LOD Scores for Chromosome 1 Markers and ADH in HC2

		LOD Score at θ =									
Locus	Distance[a]	.00	.001	.01	.05	.10	.20	.30	.40	Z_{max}	θ_{max}
D1S234	...	−6.49	−4.95	−3.81	−2.60	−1.84	−.99	−.52	−.24	−.24	.40
D1S513	.15	1.05	1.06	1.09	1.16	1.17	1.03	.73	.31	1.18	.08
D1S2830	.01	−1.02	−1.01	−.94	−.70	−.49	−.22	−.08	−.01	−.01	.40
D1S255	.11	2.13	2.13	2.15	2.16	2.09	1.75	1.24	.58	2.16	.05
D1S472	.02	2.13	2.14	2.15	2.16	2.09	1.75	1.24	.58	2.17	.03
D1S2892	.08	3.13	3.13	3.11	2.98	2.77	2.22	1.54	.73	3.13	.00
D1S2722	.02	3.13	3.13	3.11	2.98	2.77	2.22	1.53	.72	3.13	.00
D1S211	.04	−4.52	.13	1.11	1.70	1.81	1.62	1.17	.57	1.81	.10
D1S197	.08	−2.97	−.86	.15	.88	1.13	1.15	.87	.43	1.19	.15

[a] Distance between two adjacent markers in θ.

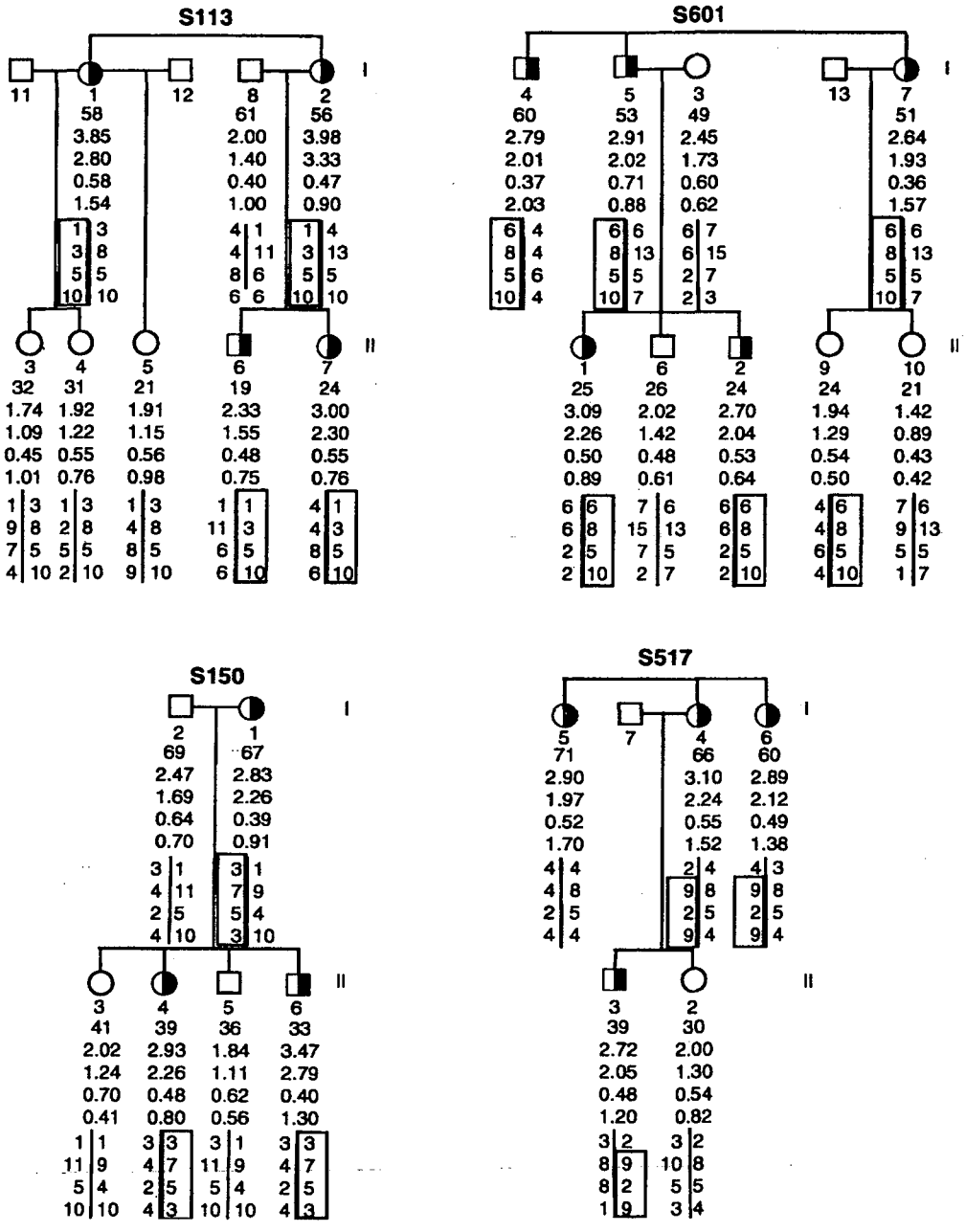

Figure 2 Segregation of chromosome 1p34.1-p32 markers in the Spanish families S113, S601, S150, and S517. Lipid values given under each family-member symbol are (*top to bottom*): age at lipid measurement levels of total, LDL, and HDL cholesterol (in grams per liter); and levels of triglycerides in (grams per liter). Haplotypes (*top to bottom*) at tel-*D1S472-D1S2892-D1S2722-D1S211*-cen markers are shown for each family member tested. Half-blackened symbols indicate affected members and unblackend symbols indicate unaffected members. The common region transmitted with the disease phenotype in each family is boxed.

chromosome 8, as well as the *APOA1-C3-A4* gene cluster on chromosome 11 and the 1q21-q23 region, both of which are associated with familial combined hyperlipidemia (Wojciechowski et al. 1991; Pajukanta et al. 1998). Because this family was clinically and biologically indistinguishable from FH or FDB families and the expected LOD score showed sufficient power in the pedigree, we used exclusion mapping to localize the disease gene. The phenotypic status of each family member was carefully and independently established by four researchers (M.D., M.K., J-P.R., and C.B.) and by other recognized French experts. To avoid spurious results from misclassification, members with borderline lipid values were scored as "unknown" in the genetic analyses (seven

Table 3

Pairwise LOD-Score Analyses for ADH and 1p Regional Markers

	Z_{max} (θ) at Marker				
Family	*D1S472*	*D1S2892*	*D1S2722*	*D1S211*	Z_{max}[a]
HC2	2.16 (.05)	3.13 (0)	3.13 (0)	1.81 (.1)	4.13
HC67	−.01 (.4)	0 (.4)	0 (.4)	0 (.4)	.86
A1	.15 (0)	−.09 (.4)	−.02 (.4)	−.09 (.4)	1.01
S150	.56 (0)	.82 (0)	.82 (0)	.82 (0)	.82
S108	nd	−.02 (.4)	−.02 (.4)	nd	.84
S113	.07 (0)	1.34 (0)	.21 (0)	.2 (0)	1.35
S517	−.03 (.4)	0 (.4)	.64 (0)	.01 (.3)	.79
S509	nd	0 (.4)	−.01 (.4)	nd	.79
S601	.09 (0)	.50 (.05)	.07 (0)	.41 (.05)	1 .61
S129	nd	−.02 (.4)	−.02 (.4)	nd	.75
S206	−.05 (.4)	.19 (.3)	−.01 (.4)	.15 (.3)	2.46
B1	−.43 (.4)	−.20 (.4)	−.2 (.4)	−.18 (.4)	1.94
NZ1	nd	−.01 (.4)	0 (.4)	nd	.50
Total Z_{max} (θ)	.17 (.3)	1.59 (.2)	1.04 (.2)	1.13 (.2)	...
	Homogeneity Admixture Text at Marker				
Total Z_{max} (θ [α])	1.43 (.1 [32%])	2.88 (0 [27%])	2.43 (0 [25%])	1.45 (.01 [46%])	...
P Value	.01	.005	.005	.11	...
	Posterior Probability of Linkage at Marker				
HC2	.985	.998	.998	.982	...
HC67	.155	.027	.004	.319	...
A1	.399	.004	.155	.154	...
S150	.625	.700	.688	.792	...
S108	.320	.014	.001	.460	...
S113	.351	.883	.351	.540	...
S517	.005	.096	.593	.344	...
S509	.320	.025	.002	.460	...
S601	.316	.533	.281	.676	...
S129	.320	.023	.009	.460	...
S206	.0004	.123	.035	.314	...
B1	.001	.001	.001	.032	...
NZ1	.320	.103	.155	.460	...

Note.—nd = not determined.

[a] Z_{max} encountered in the 500 SLINK replicates.

subjects). We chose this conservative approach despite loss of power. In the absence of established genetic parameters for this autosomal dominant disease, we assumed it to be comparable to FH or FDB. Therefore, linkage analyses were performed with a gene frequency of .002 and penetrance of .9. At *D1S2892* and *D1S2722*, the Z_{max} exceeded the threshold of 3 in the family (Z_{max} = 3.13, θ = 0; table 2), strongly supporting the hypothesis of an ADH locus at 1p. As shown in figure 1, family data also showed an affected subject (II-5) with a proximal recombination event, between *D1S2722* and *D1S211*, and an unaffected subject (III-1) with a distal recombination event between *D1S472* and *D1S2892*. Furthermore, all affected individuals shared the region flanked by markers *D1S2892* and *D1S2722*. Together these data place *FH3* in a region <9 cM, flanked by markers *D1S472* and *D1S211*. Interestingly, the subjects with no definite diagnosis (II-3, III-3, III-4, III-5, III-6, II-13, and III-15), did not carry the disease-associated two-marker haplotype. This observation supported our conservative approach. However, an 8-year-old boy (III-3), with a total-cholesterol level of 1.46 g/l and an LDL-cholesterol level of 0.72 g/l, also carried the disease-associated region. This observation explains why the Z_{max} was not reached at 1p.

In an attempt to replicate this positive linkage, we studied a second set of 12 ADH families from various countries (table 1). These families met the following clinical and biological criteria: (1) ADH phenotype; (2) normal LDL-particle receptor activity, and/or absence of mutations in the whole *LDLR* gene, and/or genetic exclusion of the *LDLR* gene; and (3) genetic exclusion of *APOB* and/or absence of mutations R3500Q and R3531C in *APOB*. Phenotypic status was established for each subject on the basis of the appropriate national reference population. Three families (S113, S150, and S517) gave two-point LOD scores, with *D1S2892* and *D1S2722* very close to their possible Z_{max} (table 3), sup-

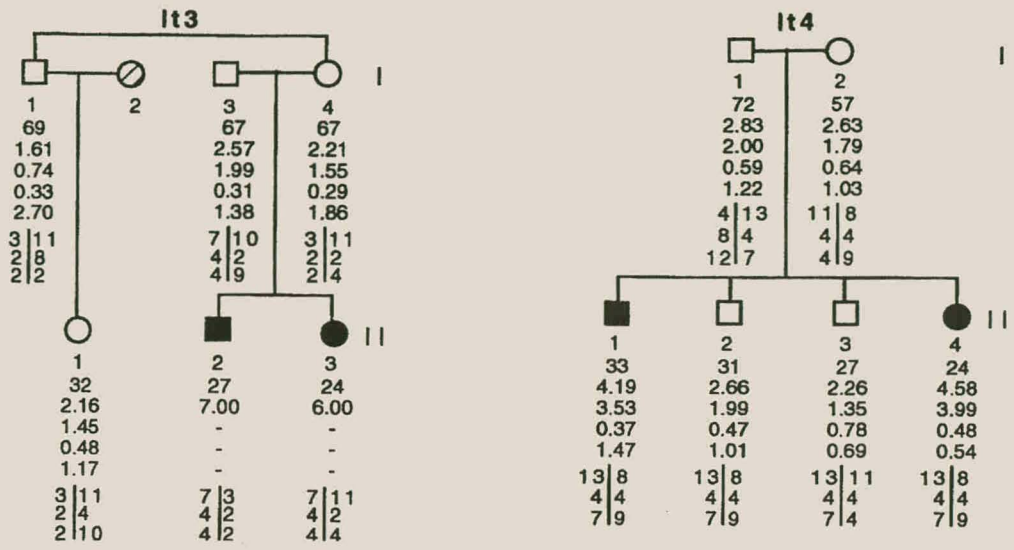

Figure 3 Segregation of chromosome 1p34.1-p32 markers in the Sardinian families It3 and It4. Lipid values given under each family-member symbol are (*top to bottom*): age at lipid measurement; levels of total, LDL, and HDL cholesterol (in grams per liter); and levels of triglycerides (in grams per liter). Haplotypes (*top to bottom*) at tel-*D1S2892-D1S2722-D1S211*-cen markers are shown for each family member tested. Blackened symbols indicate affected members, unblackened symbols indicate unaffected members, and hatched symbols indicate members with an unknown phenotypic status.

porting the localization of the *FH3* gene. In families S113 and S150, a common four-marker haplotype was carried by all the affected members. Conversely, in family S517 a positive LOD score was obtained only at *D1S2722*, but careful examination of family data did not show a conserved region shared by affected members. In family S601, LOD scores were of small value; however, all affected members shared a common region. Furthermore, this region was also shared by an unaffected 24-year-old woman (II-9; 1.94 g/l total cholesterol and 1.29 g/l LDL cholesterol). This observation could be compared with that of subject III-13 of the HC2 family. It is unlikely that both these subjects are double recombinants. However, it is highly possible that, with the criteria used for the classification of family members, the penetrance of the disease gene is not complete in children and young adults.

Admixture tests were performed on a sample comprising the HC2 family and the set of 12 ADH families. The hypothesis of genetic homogeneity was rejected against the hypothesis of heterogeneity at a significance level of 1%. These results showed that the ADH phenotype is more heterogeneous than what we at first had assumed. For the remaining ADH non-1p families, another locus, named "FH4," could be involved. We also studied two Sardinian families displaying ARH, the rare pseudohomozygous type IIa hypercholesterolemia disease with an autosomal recessive transmission. Despite the different transmission modes, we thought it was possible that some mutations in the *FH3* gene could give rise to ADH and that some others could lead to ARH, as has been reported for other diseases, such as retinitis pigmentosa (MIM 268000). As shown in figure 3, no allele identity was apparent between the affected sibs (II-2 and II-3) of family It3, and identical alleles are shared by the affected sibs (II-1 and II-4) of family It4 and their unaffected sib (II-2). These results show that, in the two ARH families, the disease is not linked to the *FH3* gene. In these families, the disease gene could be identical to either the *FH4* gene or another as yet unidentified gene.

Our data provide strong evidence for an additional locus contributing to ADH and its assignment to chromosome 1p34.1-p32. Positioning the disease locus in relation to the genetic map indicates that the *FH3* gene is located within a 9-cM interval flanked by *D1S472* and *D1S211*. These microsatellite markers map to a region that contains four candidate cloned genes, as follows: *SCP2* (sterol carrier protein 2), which encodes a lipid transport basic protein believed to facilitate the movement of cholesterol and phospholipids within the cell (Ohba et al. 1994); *MDGI/FABP3* (mammary-derived growth inhibitor/fatty acid–binding protein 3-muscle and heart), which encodes a protein that transports vehicles of hydrophobic fatty acids throughout the cytoplasm and is a candidate tumor-suppressor gene for human breast cancer, although no mutation in this gene has been reported in sporadic breast tumors (Phelan et al. 1996); *APOER2* (apolipoprotein E receptor 2), which encodes a receptor that resembles LDL and very-low-density lipoprotein receptors and is most highly ex-

pressed in human brain and placenta (Kim et al. 1997); and *EPS15* (epidermal growth factor receptor pathway–substrate), which encodes a protein that is implicated in the receptor-mediated endocytosis pathway (Wong et al. 1994). To clarify the positional relationship between these regional candidate genes and the markers linked to the *FH3* gene, we mapped them relative to the WI/MIT radiation hybrid map. The results showed that all these candidate genes are not located within the *D1S472–D1S211* interval, excluding possible identity between the *FH3* gene and one of these candidates and suggesting a novel gene for ADH on 1p34.1-p32.

To conclude, the 13 families we have identified give evidence for the existence of a greater level of genetic heterogeneity than conventionally has been assumed for ADH. Our results indirectly show that *FH3* encodes a protein, as yet unknown, whose function is important in the control of cholesterol homeostasis. Defects in this new pathway could explain the resistance to drug therapy observed among some patients with hypercholesterolemia. The identification of the *FH3* gene may help to develop new intervention strategies to limit elevation of LDL particles and prevent morbidity and mortality from premature atherosclerosis.

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Electronic-Database Information

Accession numbers and URLs for data in this article are as follows:

GENATLAS, http://citi2.fr/GENATLAS (for markers used in cytogenetic localization)
Genome Database, http://gdbwww.gdb.org (for primers used)
LDLR Mutation Database, http://www.umd.necker.fr:2004/ (for *LDLR* mutations)
Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim (for FH [MIM 143890], FDB [MIM 144010], and Retinitis Pigmentosa [MIM 268000])
WI/MIT, http://www.genome.wi.mit.edu/ (for marker and gene physical mapping)

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