Molecular diagnosis of familial hypercholesterolaemia in the diverse South African population

by

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Dedicated to Sonia, Winston, Salome and Gerhard

Jeremiah 29:11

"For I know the thoughts and plans that I have for you,
says the Lord, thoughts and plans for welfare and peace and not for evil,
to give you hope in your final outcome"

Romans 8:24

"For in this hope we were saved.
But hope which is seen is not hope.
For how can one hope for what he already sees?"

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List of abbreviations

A	adenine
ACE	angiotensin-converting enzyme
ADH	autosomal dominant hypercholesterolaemia
apo B	apolipoprotein B-100
APOB	apolipoprotein B
apo E	apolipoprotein E
ARH	autosomal recessive hypercholesterolaemia
ARMS	amplification refractory mutant system
bp	base pair/s
C	cytosine
cDNA	copy deoxyribonucleic acid
CHD	coronary heart disease
CVD	cardiovascular disease
D	deletion
del	deletion
DGGE	denaturing gradient gel electrophoresis
DNA	deoxyribonucleic acid
EGF	epidermal growth factor
FDB	familial defective apolipoprotein B-100
FH	familial hypercholesterolaemia
G	guanine
HDL	high density lipoprotein
HDLC	high density lipoprotein cholesterol
HEX-SSCP	heteroduplex-single strand conformation polymorphism
I	insertion
ins	insertion
kb	kilobase
LDL	low density lipoprotein
LDLC	low density lipoprotein cholesterol
LDLR	low density lipoprotein receptor
Lp(a)	lipoprotein(a)
MHFTR	methylenehydrofolate reductase
MED-PED	Make Early Diagnosis to Prevent Early Deaths in MEDical PEDigrees
MI	myocardial infarction
mmol/l	millimoles per litre
mRNA	messenger ribonucleic acid
PCR	polymerase chain reaction
SD	standard deviation
SSCP	single strand conformation polymorphism
T	thymine
TC	total cholesterol
TG	triglycerides
VLDL	very low density lipoprotein
yrs	years
XMTA	xanthomata

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CHAPTER 1

INTRODUCTION

The genetic basis of primary hypercholesterolaemia, diagnosis and treatment

Primary hypercholesterolaemia, or the elevation of plasma low density lipoprotein (LDL) cholesterol which is not secondary to environmental, dietary or other underlying disease, is a relatively common condition that has been associated with the development of premature cardiovascular disease (CVD) (Ose 1999). Monogenic hypercholesterolaemia occurs when the increase in circulating LDL cholesterol is caused by molecular defects in a single gene. Plasma lipoprotein levels are determined by the interaction of heritable and environmental factors.

1. AUTOSOMAL DOMINANT HYPERCHOLESTEROLAEMIA

Autosomal dominant hypercholesterolaemia (ADH) is an inherited disorder of lipid metabolism, the hallmark of this phenotype being the elevation of circulating LDL cholesterol particles, the presence of tendon xanthomata, and premature mortality from cardiovascular complications (Goldstein and Brown 1989). Until recently it was generally assumed that familial hypercholesterolaemia (FH) (Goldstein et al. 1995) and familial defective apolipoprotein B-100 (FDB) (Innerarity et al. 1990) are the only causes of monogenic primary hypercholesterolaemia. The prevalence of both diseases is approximately 1/500 in most Caucasian populations (Goldstein et al. 1995). The FH phenotype is characterised by mutations in the low density lipoprotein receptor (LDLR) gene (Hobbs et al. 1992), whereas FDB is caused by mutations in the apolipoprotein B (APOB) gene (Soria et al. 1989). Impaired removal of LDL is the primary defect in these related syndromes. In FH the normal function of the LDL receptor is disrupted, while in FDB the ligand apolipoprotein B-100 (apo B-100) is abnormal. Each ß-very low density lipoprotein (VLDL) particle contains multiple copies of apolipoprotein E (apo E),

while LDL has only one apo B molecule. Since the function of LDLR is to internalise ß-VLDL through binding of apo B, it is clear that a mutant receptor will have a stronger impact on cholesterol homeostasis than defective apo B-100. In FH the clearance of both ß-VLDL and LDL will be impaired, resulting in severe hypercholesterolaemia in the majority of affected patients. As for FDB, the catabolism of apo E-containing ß-VLDL is still intact and only defective LDL particles would contribute to the elevation of plasma cholesterol levels (Brown and Goldstein 1986).

Recently, a third major locus for ADH, designated FH3, was mapped to chromosome 1p34.1-p32 (Varret et al. 1999). Linkage analysis indicated that only ~27% of the clinically diagnosed non-LDLR / non-APOB FH families studied are linked to FH3, thus leading to the proposal of a fourth major locus for ADH (Varret et al. 1999). The genetic heterogeneity of ADH was further underlined when the involvement of LDLR and APOB gene defects was excluded in clinically diagnosed FH families from Utah (Haddad et al. 1999).

2. AUTOSOMAL RECESSIVE HYPERCHOLESTEROLAEMIA

Several reports on an autosomal recessive form of hypercholesterolaemia were noted (Sirtori et al. 1991, Harada-Shiba et al. 1992, Zuliani et al. 1999). In these patients, the hypercholesterolaemic phenotype was absent in both parents. Although patients presented with marked increased levels of LDL plasma cholesterol they exhibit normal levels of LDL particles and receptor activity. In 1999 a new lipid disorder, familial recessive hypercholesterolaemia, was proposed (Zuliani et al. 1999). The disease phenotype resulted from a marked reduction of *in vivo* LDL catabolism caused by a selective reduction of hepatic LDL uptake. Ciccarese and colleagues (2000) mapped this disorder, designated as ARH1, to chromosome 15q25-q26.

3. FAMILIAL DEFECTIVE APOLIPOPROTEIN B-100

Familial defective apolipoprotein B-100 (FDB) is a co-dominant autosomal lipid disorder caused by mutations in the binding region of the apolipoprotein B (APOB) gene located on chromosome 2p23-p24 (Law et al. 1985, Brown and Goldstein 1986, Huang et al. 1986). This gene spans 43kb (Knott et al. 1985) and encompasses 29 exons and 28 introns (Blackhart et al. 1986). The APOB cDNA contains 13 860 nucleotides, which are translated into 4 560 amino acids (Cladaras et al. 1986). Exon 26 of the APOB

gene covers more than half (7 572 bp) of the length of the cDNA and encodes a region that is involved in binding to the LDL receptor (Milne et al. 1989). Three mutational events in exon 26 of the APOB gene underlying the FDB phenotype have been described. This causes a defective apolipoprotein, which is the main constituent peptide of LDL and serves as the ligand for receptor-mediated uptake of LDL, to bind with reduced affinity to the receptor molecule (Brown and Goldstein 1986). Mutation R3500Q entails a base substitution of glutamine to arginine at codon 3500 (Soria et al. 1989) (prevalence ~1:500), while mutation R3531C (prevalence ~1:3000) results in a base change of arginine to cysteine at codon 3531 of the APOB gene (Pullinger et al. 1995, 1999). The third, more recently described APOB defect also occurs on codon 3500 and is designated as R3500W (Gaffney et al. 1995).

FDB seems to be quite common (1:400 to 1:700) in the Netherlands (Defesche et al. 1991), Switzerland (Miserez et al. 1994), North America (Innerarity et al. 1990) and Belgium (Kotze et al. 1994). A lower prevalence of this disease has been reported south of the Alps, in Russia, in Scandinavia and in Denmark (Hansen 1998), with an apparent absence in Finland (Hämäläinen et al. 1990) and Japan (Hosking et al. 1991). FDB is rare in the South African population (Rubinsztein et al. 1994, 1995) and is apparently absent in non-Caucasian populations (Tybjærg-Hansen and Humphries 1992), most likely as a result of under representation of mutation R3500Q in South African settler populations who are mainly of European descent (Rubinsztein et al. 1995). Since this mutation originated in Europe, after racial diversification (Ludwig and McCarthy 1990), any FDB mutation that might have arrived in the Coloured gene pool from European ancestors would have been diluted. More recently, Loubser and colleagues (1999) reported the presence of molecular lesion, R3500Q, in a South African hypercholesterolaemic patient of mixed ancestry (Coloured). Double heterozygosity for both FDB and FH was reported in a large Afrikaner-English South African kindred (Rubinsztein et al. 1993b, Raal et al. 1997b). FH-causing mutation, D206E, and FDB-causing mutation, R3500Q, were identified in the index patient, while a family member was a "complex" heterozygote for LDLR gene defects, D206E and V408M, as well as FDB mutant allele, R3500Q. These hypercholesterolaemics presented with clinical features that are intermediate in severity between heterozygous and homozygous FH.

Variability in clinical expression of FDB does exist and may complicate disease diagnosis (Myant et al. 1991, März et al. 1992, Gallagher and Myant 1993). This lipid disorder is characterised by a relatively mild phenotype, comparable to polygenic

hypercholesterolaemia (Tybjærg-Hansen and Humphries 1992, Miserez and Keller 1995) or the receptor-defective FH phenotype (Kotze et al. 1994). The phenotypic expression of FDB homozygous patients is less severe than in patients with homozygous FH (Schaefer et al. 1997).

4. FAMILIAL HYPERCHOLESTEROLAEMIA

Familial hypercholesterolaemia (FH) is a co-dominant autosomal disorder of lipid metabolism, characterised by elevated serum LDL cholesterol levels, accelerated atherosclerosis and premature coronary heart disease (CHD) (Goldstein et al. 1995).

4.1 The LDLR gene

The FH phenotype results from defective catabolism of LDL, which is caused by mutations in the LDLR gene located on chromosome 19p13.1-p13.3 (Goldstein et al. 1995). The LDLR gene spans 45kb and encompasses 18 exons and 17 introns (Südhof et al. 1985), of which a 5.3kb mRNA molecule is transcribed (Yamamoto et al. 1984). This gene is a highly mutagenic locus and contains twice as many Alu-repeats as the average human gene, and these represent hotspots for recombination events. The inherent nature (high CpG dinucleotide content) of exon 4 of this gene renders itself as another hotspot region for mutational events due to frequent transition of cytosine to thymine at CpG dinucleotides containing methylated cytosines (Hobbs et al. 1990). The LDLR gene is a mosaic of exons shuffled to and from other genes during evolution. Thirteen of the 18 exons encode protein sequences that are homologous to proteins encoded by other genes (Südhof et al. 1985). To date, more than 600 LDLR gene defects (http://www.ucl.ac.uk/fh and http://umd.necker.fr) (Day et al. 1997, Varrett et al. 1997, 1998) underlying FH have been identified, with an estimated prevalence of 1 in 500 for heterozygotes and 1 in a million for homozygotes in most European populations (Goldstein et al. 1995).

4.2 The LDL receptor

The LDLR gene encodes an 860 amino acid precursor molecule. After maturation a membrane-bound glycoprotein consisting of 839 amino acids is released at the cell surface (Yamamoto et al. 1984, Südhof et al. 1985). The mature LDL receptor protein

consists of five distinct functional domains (Goldstein et al. 1995). These are the ligand-binding domain encoded by exons 2 to 6, the epidermal growth factor (EGF) precursor homology domain encoded by exons 7 to 14, the carbohydrate side chains (O-linked sugars) encoded by exon 15, the membrane spanning domain encoded by exon 16 and a part of exon 17, and the cytoplasmic tail encoded by a part of exon 17 and exon 18 with its N-terminal signal sequence (21 hydrophobic amino acids) on the outside of the plasma membrane and a C-terminal sequence inside the cell cytoplasma (Russell et al. 1984).

LDLR gene defects have been grouped into five distinct classes, depending on the resulting disturbance in function. Class I mutations or null alleles produce no detectable receptor. These mutations often result from large deletions of the promoter region or by nonsense mutations and deletions that create premature stop codons. Class 2 mutations or transport-deficient alleles result in receptors that are either slowly transported from the endoplasmic reticulum (ER) to the Golgi apparatus or retained in the ER where the particles are eventually degraded. Class 3 mutations or binding-deficient alleles exhibit impaired ligand binding. Class 4 mutations or internalisation-defective alleles involve the cytoplasmic tail. The mutant alleles produce receptors that bind LDL normally, but fail to cluster in coated pits and therefore fail to transport LDL particles into the cell. Class 5 mutations or recycling-deficient alleles fail to separate from bound ligands after being internalised in endosomes. Trapped in the cell these molecules are rapidly degraded (Hobbs et al. 1990, Rubinsztein et al. 1994).

4.3 Phenotypic expression and variability of FH

Homozygous FH is a chronic and debilitating disease characterised by vastly raised cholesterol levels, the presence of tendon xanthomata, premature arteriosclerosis and a high morbidity from cardiovascular complications, usually starting as early as the first decade of life. Angina pectoris, myocardial infarction (MI) or sudden death resulting from CHD, usually manifests between the ages 5 and 30 (Goldstein and Brown 1989, Yamamoto et al. 1989). Clinical expression is milder in FH heterozygotes where half the normal LDLR activity is usually expressed, and consequently results in an approximate twofold increase of plasma LDL cholesterol levels (above the 95th percentile for age and gender), the development of tendon xanthomata and CHD, which is 25 times more common in heterozygotes than in unaffected individuals (Myant 1990, Goldstein et al. 1995).

Variation in phenotypic expression of FH, even in homozygous FH, is well documented, despite the inherited nature of this disease (Kotze et al. 1993a, 1993b, Moorjani et al. 1993, Gudnason et al. 1994, Graadt van Roggen et al. 1995, Pimstone et al. 1998, Yamamoto et al. 1989). Although significant differences in LDL cholesterol levels were demonstrated in adult Afrikaner FH heterozygotes with either receptor-defective (D154N and D206E) or receptor-negative (V408M) LDLR gene defects (Kotze et al. 1993a, Graadt van Roggen et al. 1995), no significant differences were observed in children (Kotze et al. 1998b). Other influences, apart from the LDLR gene defect, therefore appear to be more important in determining lipid levels in FH heterozygous children than in adults.

CHD is more prominent and more frequent in homozygous FH patients with molecular defects resulting in LDL receptor-negative phenotypes than those with receptor-defective phenotypes (Moorjani et al. 1993). Differences in plasma cholesterol concentrations are reflected in the severity of CHD expression. Although the phenotypic expression of CHD and coronary deaths are related to LDLR status (Goldstein and Brown 1982, Yamamoto et al. 1989) the contribution of other modifier genes in the phenotypic expression of FH should be considered.

South African Afrikaans-speaking whites (Afrikaners) have one of the highest death rates from CHD in the Western world, and FH can be considered a major contributing factor to development of this condition (Wyndham 1978, Seftel et al. 1980, Pretorius 1983). In the Afrikaner population the mortality rate in men in the age group 25 to 34 years, is twice as high as reported in North America, more than 3 times higher than reported in Finland and more than 7 times higher than in Sweden (Wyndham 1978). Males diagnosed with FH have up to a 40-fold increased risk of premature CHD and, if untreated, their life expectancy is reduced by 20 to 30 years. These males usually die of CHD between the ages of 35 and 55, and women between 55 and 75 years (Williams et al. 1993a). In the USA and Western Europe CHD is responsible for at least 30% of the total mortality rate in men aged 45 to 75 years (Wyndham 1978, Utermann 1983). The high mortality rate due to CHD and coronary deaths, particularly in the South African Afrikaner and Jewish populations (Walker 1963), have important health implications.

4.4 The possible role of modifier genes in the clinical expression of FH

Gene-gene and gene-environment interaction determines risk factors or protection with respect to the development of CHD. Molecular lesions in certain genes, like the LDLR gene, may have a major effect, while polymorphisms in other genes play a moderate role in the development of CHD in FH patients. It has been suggested that FH contributes only to a small proportion of the risk for coronary death in the population at large, since merely 5% of patients with atherosclerotic disease have FH (MacCluer and Kammerer 1991).

Hobbs and colleagues (1989) presented strong evidence supporting the notion that a lipid-lowering gene exists. They identified an FH family that presented with lower than expected LDL cholesterol concentrations in certain affected individuals. This was further substantiated by the identification of a 5kb deletion in the LDLR gene in an extended French-Canadian kindred with normal LDL cholesterol levels (Sass et al. 1995). Recently, Knoblauch and co-workers (2000) performed genotype-phenotype correlation studies in a large Arab family with FH and reported an LDLR gene defect which results in extremely low receptor activity. Despite this, the affected family members presented with normal LDL cholesterol levels. After linkage-analysis, they mapped a putative cholesterol-lowering gene to chromosome 13q.

Genes with only a slight effect can be clinically important in combination with other genes or life-style factors (Abbate et al. 1998, Berg 1998). Important risk factors in FH determined by genes and environmental interaction include lipoprotein (a) (Seed et al. 1990, Wiklund et al. 1990, Scholtz et al. 2000a), hyperhomocysteinaemia (Peeters 1997, Berg 1998, Scholtz et al. 2001) and angiotensin converting enzyme (Petrovic et al. 1996, O'Malley et al. 1998, 1999).

4.4.1 Lipoprotein (a)

Lipoprotein (a) [Lp(a)] has gained much attention for its role in the pathogenesis of CHD. Although elevated levels of Lp(a) is an established risk factor for CHD, particularly in FH patients with a concominant increase in LDL cholesterol (Seed et al. 1990, Wiklund et al. 1990, Lingenhel et al. 1998, Real et al. 1999), contradiction exists (Mbewu et al. 1991, Tatò et al. 1993, Bowden et al. 1994, Ferrières et al. 1995). Variation at the apo (a) locus on chromosome 6 is to a large extent responsible for the observed variation in plasma Lp(a) levels between individuals (Boerwinkle et al. 1992).

Lp(a) is a modified LDL particle of human plasma (Real et al. 1999) and raised LDL levels due to molecular lesions underlying the FH phenotype may subsequently lead to the elevation of Lp(a) levels in the plasma (Utermann et al. 1989, Wiklund et al. 1990). The function and metabolism of Lp(a) remains to be established. It has been suggested that Lp(a) competes with LDL for LDL receptors (Hoffmann et al. 1990) and that only 25% of Lp(a) is catabolised via the LDL pathway (Snyder et al. 1992). Lp(a) may also have a function in wound healing by binding fibrin at wound sites (Brown and Goldstein 1987).

No treatment is really efficient to lower Lp(a) level and a good observance is required for lifelong treatment (Foubert et al. 1997). FH patients is expected to have an incremental risk for CHD due to the potentiation of hypercholesterolaemia and elevated Lp(a) values, indicating the additional effects of two different loci implicated in the pathogenesis of CHD. The proposed gene-gene interaction may partly explain the phenotypic variation in FH (Real et al. 1999).

4.4.2 Homocysteinaemia

Hyperhomocysteinaemia is an established independent risk factor for the development of CHD (Frosst et al. 1995, McCully 1996). A common methylenetetrahydrofolate reductase (MTHFR) gene polymorphism, 677C→T (Frosst et al. 1995), is believed to modulate risk of CHD through regulation of homocysteine metabolism (Frosst et al. 1995, Kluijtmans et al. 1996, Boers 1997, Loktionov et al. 1999). More recently, a second sequence variant in the MTHFR gene located on chromosome 1p36.1 (Goyette et al. 1994), mutation 1298A→C, has been identified (Girelli et al. 1998). Although this molecular lesion is not associated with elevated plasma homocysteine levels or lower plasma folate concentration, combined heterozygosity of both these mutations result in increased thermolability of the enzyme and elevated homocysteine levels. The possible role of mutation 1298A→C in CHD has not yet been defined, but it is indicated as an additional risk factor for neural-tube defects (Van der Put et al. 1998). Affected patients with mildly elevated homocysteine levels may be treated with folate supplements (Kang et al. 1991).

Racial and geographical differences attribute to the heterogeneous distribution of these molecular lesions and may partly explain the differences in CHD risk in different ethnic groups from various geographical parts of the world (Franco et al. 1998, Loktionov et al. 1999, Ubbink et al. 1995). The MTHFR 677C→T mutation does not

constitute an important CHD risk factor in black South Africans (Ubbink et al. 1995, Loktionov et al. 1999, Scholtz et al. 2001). South African blacks seem to metabolise homocysteine more effectively than whites and may therefore be protected against CHD by an effective homocysteine metabolism (Ubbink et al. 1995). Moderate hyperhomocysteinaemia is associated with a parental history of CHD in children with FH, and homozygosity for mutation 677C→T appears to be more frequent in these children (Tonstad et al. 1997).

It is suggested that these genetic alterations are not 'per se' involved in the pathogenesis of CHD, but that it may become of clinical significance in the presence of other genetic, dietary or environmental factors (Abbate et al. 1998).

4.4.3 Angiotensin-converting enzyme

The insertion-deletion (ID) polymorphism of the angiotensin-1-converting enzyme (ACE) gene (Rigat et al. 1990, Tiret et al. 1992), located on chromosome 17q23 (Mattei et al. 1989), has been implicated as a possible pathogenic factor in the development of CHD. Controversy exists on the possible role of this genetic polymorphism and various reports have shed serious doubts on the significance thereof (Butler et al. 1997, Schunkert 1997, Staessen et al. 1997). Half of the phenotypic variation in the plasma ACE levels can be attributed to the segregation of the ACE I/D polymorphism, residing in intron 16 on the ACE gene. The ACE DD genotype is associated with almost double the levels of circulating enzyme (Rigat et al. 1990). ACE converts angiotensin I to the vasoconstrictor angiotensin II, and catalyses the degradation of bradykinin to inactive peptides with consequential proliferation of the smooth muscle cells and vasospasm (Cambien et al. 1992).

Studies on the significance of the ACE polymorphism in FH patients demonstrated an association of the D allele with an increased risk for CHD (Petrovic et al. 1996, O'Malley et al. 1998, 1999). The effect of the DD genotype appears to be small and its clinical manifestations rather heterogeneous, which may partly be explained by geographical and racial differences. The clinical significance of the genetic marker may be gender-dependent in some populations (O'Malley et al. 1998, 1999).

The clinical consequences of an increased risk for CHD associated with the ACE DD genotype in an already high-risk population are quite severe. The incidence of CHD in FH heterozygous males with the ACE DD genotype is 2.2 times higher than those

who have the ACE ID/II genotypes (O'Malley et al.1998). Petrovic and colleagues (1996) reported an excess of the ACE DD/ID genotypes in CHD-positive versus CHD-negative FH patients. Patients with FH as well as the ACE DD genotype may therefore be at increased risk for developing CHD. Identification of modifier genes that may influence the clinical expression of common single gene disorders, like FH, is of fundamental importance for genetic counselling and treatment strategies.

More recently, the ACE gene has been associated with both negative and positive effects with respect to survival. The DD genotype of the ACE polymorphism appears to be associated with longevity (Galinsky et al. 1997), despite the fact that the deletion allele appears to increase the risk of CHD. Interestingly, the ACE ID and II genotypes were associated with increased susceptibility for Alzheimer disease (Narain et al. 2000).

5. The diagnosis, treatment and management of FH

The clinical diagnosis of FH is based on the elevation of low density lipoprotein (LDL) cholesterol levels, the presence of tendon xanthomata and/or a family history of premature coronary heart disease (CHD). Homozygous FH is characterised by severe clinical manifestation of tendon xanthomata and atherosclerosis, usually associated with premature death during childhood (Goldstein et al. 1995). The clinical picture develops through accumulation of cholesterol-rich lipoproteins in plasma, which results from the impaired uptake and degradation of LDL by a mutant receptor. Depending on the nature of genetic defect(s) and environmental influences, the severity of clinical consequences of FH may vary considerably (Thompson et al. 1989, Jeenah et al. 1993, Kotze et al. 1993a, 1993b, Pimstone et al. 1998). This, together with the fact that certain features of FH are non-specific, renders an unequivocal diagnosis based solely on biochemical and clinical data a troublesome task. Plasma cholesterol values frequently overlap between FH heterozygotes and normal individuals, and particularly in children, lipid levels may not be diagnostic of FH.

Advances in molecular biology now allow accurate diagnosis of FH, which facilitates clinical management of patients and may provide them with a longer life expectancy. Mutation analysis furthermore allows differential diagnosis of FH and FDB (Soria et al. 1989, Innerarity et al. 1990, Wenham et al. 1991), which share similar clinical features. This is an important consideration, since FDB appears to be a less severe condition in comparison with FH, and might therefore require a less aggressive

treatment strategy. Genetic and environmental factors may confer additional atherosclerotic risk to heterozygous FH patients and may play a role in the expression of FH disease-causing mutations. Extensive research is therefore warranted to identify loci that may modify CHD risk in FH patients, in order to eventually develop a multilocus assay that may complement the use of traditional risk factors in prediction of clinical outcome. It has been estimated that approximately 50% of patients with cardiovascular disease do not have any of the established risk factors, such as elevated plasma cholesterol levels, hypertension, smoking, obesity and diabetes mellitis. Identification of genetic risk factors that can be reduced by appropriate therapeutic intervention may lead to effective treatment targeted at the cause of the disease. In hypercholesterolaemics who do not respond well to current lipid-lowering drug treatment, delineation of molecular mechanism(s) underlying the clinical manifestation of FH may allow individualised treatment and avoidance of adverse side effects due to inappropriate treatment. It is envisaged that the identification of novel genes underlying locus heterogeneity in primary hypercholesterolaemia would in future facilitate clinical management of affected patients.

The benefits of treating heterozygous FH patients with lipid lowering drugs are well recognised and are cost effective as a primary preventive treatment (Goldman et al. 1993). With adequate and well managed cholesterol lowering, regression of skin and tendon xanthomas are observed (Illingworth et al. 1990). Kane and colleagues (1990) reported a regression of coronary atherosclerosis and reduction in CHD in both sexes with aggressive cholesterol lowering strategies. Strategies for treating patients with FH are at present directed at lowering the plasma level of LDL. In heterozygous FH this is accomplished through administration of drugs that stimulate the expression of LDL receptor from the normal allele. This therapeutic approach is not effective in homozygous FH, especially those that only retain less than 2% of residual LDL receptor function. In these patients chronic plasmapheresis therapy and a more direct approach, liver transplantation, to correct the deficiency of hepatic LDL receptor (Wilson et al. 1992), is necessary to accomplish cholesterol lowering.

5.1 Family-based screening approach

It is estimated that of the 10 million people affected with FH worldwide the vast majority is unaware of their increased risk for developing CHD. Approximately 5% of all patients under the age of 45 years who suffered a myocardial infarction (MI) carry this trait

(Wilson et al. 1992). The vast majority of the calculated 80 000 FH heterozygotes in South Africa have not yet been identified and of the expected 200 homozygotes, less than 100 patients have been diagnosed as having FH (Kotze et al. 1998a). Approximately 10% of white South Africans with an MI before age 60 have heterozygous FH.

The MED-PED FH (Make Early Diagnosis, Prevent Early Death in Medical Pedigrees with Familial Hypercholesterolemia) Project is an international effort aimed at diagnosing patients and introducing proper treatment regimes to prevent premature coronary deaths. The rationale of a family-based mutation screening approach as proposed by the MED-PED initiative, firstly involves identification of FH-causing mutations in index patients. Secondly, follow-up DNA screening in family members is performed to identify high-risk mutation-positive cases. This will assure identification and treatment early in life and provide the opportunity for appropriate genetic counselling. The international MED-PED initiative, established to promote tracing of defective genes in extended pedigrees, can be extended to many other common diseases in South Africa (Kotze and Callis 1999, Appendix 1).

5.2 Clinical versus molecular diagnosis

It has previously been demonstrated that although clinical diagnosis compliments DNA diagnosis of FH, the latter appears to be more accurate (Koivisto et al. 1992a, Kotze et al. 1992). Utilisation of standard blood cholesterol criteria may lead to underdiagnosis of at-risk relatives of diagnosed FH index patients, and overdiagnosis of FH in the general population (Williams et al. 1993b). In a comparative study performed over a five-year period in the Netherlands, it was shown that approximately 18% of cases, either affected or unaffected, would have been misdiagnosed based solely on cholesterol measurements (Umans-Eckenhausen et al. 2001). At the time of molecular diagnosis only 39% of FH heterozygotes received some lipid-lowering treatment, and 12 months later this had increased to 93%. Mutation screening in families proved to be highly effective in identifying patients with FH. Most of these patients sought treatment after diagnosis to lower their risk of premature CHD.

In a recent study performed in the South African population, the molecular diagnosis of FH was evaluated against routinely used biochemical parameters in 790 at-risk relatives of 379 index cases (J Vergotine et al. 2001a). The relevant FH-causing mutations were detected in 338 close relatives and excluded in 452 subjects. It was

shown that 15.6% of at-risk family members of molecularly-characterised FH index cases may be misdiagnosed when total cholesterol concentration at the 80th percentile for age and gender (Rossouw et al. 1985) is used as a biochemical cutoff point for a diagnosis of FH, compared to 12.4% using the 95th percentile. In total, 16/150 (10.7%) relatives with an FH mutation were falsely classified as normal (negative predictive value of 89.3%), while 53/293 (18.1%) without the mutation were falsely classified as FH heterozygotes (positive predictive value of 81.9%). The sensitivity and specificity of FH diagnosis according to TC values (80th percentile) were therefore 89.3% and 81.9%, respectively.

Most heterozygous FH patients do not present with cholesterol deposits in the skin and tendons, which complicates disease diagnosis and consequently preventive treatment (Goldstein et al. 1995). Since it is firmly established that FH is underdiagnosed, it should be possible to remedy this situation. Weak points in the diagnosis of FH may include the low response rate of the general practitioners, the possible errors associated with self reporting of family relationships, and the use of absolute cut-off points for cholesterol for diagnosis instead of DNA testing (Kastelein 2000). Since this disorder begins early in life, has severe consequences, and can be treated effectively the importance of an accurate diagnosis, proper treatment and follow-up should be stressed (Illingworth and Bacon 1989).

5.3 Presymptomatic and prenatal diagnosis

The majority of children with FH present with elevated plasma cholesterol levels at an early age and are at risk of developing CHD in adulthood. In addition, patients with homozygous FH may experience coronary events in childhood and rarely survive beyond the age of 20 years. Prenatal diagnosis of this severely debilitating condition justifies termination of the pregnancy, while there are no grounds for such a procedure in a foetus found to be heterozygous for FH. In heterozygous FH the clinical symptoms are of late onset and CHD risk can be modified by life-style factors and drug therapy. Genetic diagnosis of FH in childhood remains important, since the use of a novel technique to identify endothelial cell dysfunction in arteries in the forearm has suggested that such damage is present in children with FH as young as 10 years old (Celermajer et al. 1992), and this appears to be reversible by lipid-lowering treatment (Drexler et al. 1991). It has been shown repeatedly that plasma lipid levels of FH patients overlap with those in the general population, particularly in childhood where the

use of lipid levels alone may lead to misdiagnosis in approximately 10% of children (Leonard et al. 1997, Kotze et al. 1998b). Analysis of 221 South African children, from 85 families with FH, demonstrated the potential diagnostic value of mutation screening in a population with an enrichment for certain mutations (Kotze et al. 1998b). The sensitivity and specificity of FH diagnosis according to TC values were 93% and 98%, respectively, compared to unequivocal genetic diagnoses of FH in 116 children following mutation screening. Within FH families various genetic and environmental factors are shared, and therefore the plasma cholesterol levels have in general differed significantly between affected and unaffected children. This diagnostic advantage when using plasma cholesterol level, however, does not apply to the population as a whole. It has been noted that children with FH-related mutations may initially present with lipid levels within the normal range (Kessling et al. 1990), with elevated levels only developing at a later age, but the frequency of this phenomenon is unknown.

The first documented prenatal diagnosis of FH was reported in 1978 and involved the use of functional assays for quantitative assessment of LDLR activity in cultured amniotic fluid cells (Brown et al. 1978). The significance of DNA screening in a population with a high rate of consanguinity and/or the presence of a founder effect was underlined by the prenatal molecular diagnosis in a Christian-Arab family (Reshef et al. 1992). Prenatal diagnosis, aimed at the detection of homozygous cases, is important in the Afrikaner population where the prevalence of FH has been increased to 1:70 due to a founder effect (Steyn et al. 1996), since the likelihood of unions which could result in FH homozygous offspring is significantly increased (Seftel et al. 1980). The first documented prenatal FH diagnosis in South Africa was offered to an Afrikaner couple, both partners heterozygous for the same mutation. Their firstborn was diagnosed with heterozygous FH and the second child with homozygous FH. The absence of the disease-causing mutation, D206E, was confirmed on both cultured and uncultured amniotic cells in the foetus and a baby boy with plasma cholesterol levels within the normal range was subsequently born (Vergotine et al. 2001b). Although there are well founded reasons for testing asymptomatic children and adolescents for FH, ethical concerns over issues such as informed consent and disclosure to the child should be addressed. The test should only be conducted if it is in the best interest of the child, as is the case in FH since early treatment can prevent irreversible damage, otherwise testing should be postponed until adulthood.

5.4 Ethical issues

Ethical issues associated with genetic testing are of major concern since such information may impact on an individual's ability to obtain life or health insurance, or lead to unwarranted discrimination for employment or career development. The emotional trauma engendered by the need to consider a termination for homozygous FH and to decide whether or not to have the test in the first place must not be ignored. Informing parents of their reproductive choices places a considerable burden on them, and counselling and support will be needed whatever the decision.

Genetic counselling should include the provision of accurate, full and unbiased information that individuals and families require to make decisions in an empathetic relationship that offers guidance and assist people to work towards their own decisions. Counselling must be non-directive and non-coercial which means that the counsellor may not direct the deliberations or decisions of a counsellee according to the views or values of the counsellor. The information provided should enable the counsellee to take a decision from his / her own frame of reference. It is of critical importance that any genetic information be treated as confidential and is not made available to any third party without permission from the patient. The following issues should be explained to each patient before blood samples are collected for genetic testing:

- FH is a treatable autosomal co-dominant disease that runs in families.
- if a mutation is found, this information could be used for unequivocal diagnosis in first-degree relatives, particularly in combination with a cholesterol test which could then provide a clear diagnosis.
- the chance of finding mutations using current technology is not 100%, but that the majority of mutations can be detected.
- this information will be provided if the patient would like to know the outcome of the test, with an explanation of the implications.
- only after mutation data became available will the issue of DNA screening in relatives be pursued.
- access to treatment options will not be influenced by a decision about taking up DNA testing.

The main ethical dilemma arises from a conflict between the right of the individual to personal privacy on the one hand, and the interest of family members on the other hand, to be made fully aware of available information which would play a part in making

important life decisions. In the context of genetic screening, where large numbers of tests are being undertaken, this may be recorded in the form of a genetic register or similar database. Special consideration has therefore to be given to the implementation for security of these grouped results.

The negative impacts of genetic screening can be separated into two categories of harm. The first is the effect on the personal choices and mental well being of the individual, and the second is the effect on the interaction of that individual with society at large. Obvious emotional burdens of carrying a diagnosis of heterozygous FH are the fear of heart disease and the necessity of adherence to dietary and possibly lipid-lowering drug treatment to reduce the risk of CHD. However, since FH is treatable, this is considered a reasonably acceptable prospect compared with less modifyable conditions or incurable genetic diseases. In order to address these issues, the attitude of apparently healthy subjects diagnosed with heterozygous FH was determined by means of a questionnaire involving patients who attended a lipid clinic in Denmark (Andersen et al. 1997). A relatively large proportion of respondents (30-40%) expressed anxiety, followed by fear for CHD (37%) and diminishing of well-being (7-13%) due to the awareness of heterozygous FH. Although a small minority (4-6%) regretted their knowledge of the FH diagnosis, the majority (84%) were in favour of family screening for heterozygous FH, and seemed to approve (89%) of detecting the risk of modifiable disease. These findings should encourage programs for systematic screening for heterozygous FH in families that are identified through an index patient with FH. However, in order to limit negative reactions the diagnosis should be accompanied by individual counselling about risk, modifying factors, and treatment possibilities.

In a study performed in a large group of Finnish children and young adults with FH to determine their cardiovascular risk (Porkka and Viikari 1994), the following reasons were given for not recommending universal screening strategies, although the importance of early diagnosis of FH was stressed: (i) the limited predictive power of serum lipid levels on an individual level, (ii) the limited knowledge on the safety of interventive measures on the growing child, and (iii) the limited knowledge on the ethical aspects of screening. Humphries and colleagues (1997), on the other hand, indicated that molecular diagnosis is especially useful in children, in whom plasma lipid levels may not always be diagnostic. DNA screening is also highly appropriate when clinical symptoms characteristic of FH or family history are equivocal or absent, and for the detection of FH causative mutations in close family members where there is a family

history of premature CHD. Based on current data, it can therefore be accepted that the use of an unequivocal DNA diagnostic procedure in combination with measurements of plasma cholesterol levels to test for FH is ethically acceptable, is unlikely to be associated with a significant psychological impact, and is likely to be associated with reduction in morbidity (Humphries et al. 1997).

6. Taking advantage of the founder effect in the African context

In most countries the molecular heterogeneity of FH complicates molecular diagnosis and/or prevents the application of standard genetic tests. However, DNA-based diagnosis can be performed cost-effectively in genetically isolated populations, with an enrichment of specific disease-causing mutations. In the Afrikaners (Kotze et al. 1991), French-Canadians (Leitersdorf et al. 1990), Christian Lebanese (Reshef et al. 1992), Finns (Aalto-Setälä 1988) and Ashkenazi Jews of Lithuanian ancestry (Meiner et al. 1991), founder effects have resulted in an extremely high prevalence of specific disease-related mutations (Table 1.1). In these populations specific mutations are present in groups of presumably unrelated persons. These so-called founder effects probably arise when a small founder group, which by chance includes a number of individuals with a defective gene, undergoes great numerical expansion while remaining relatively isolated due to geography, language, religion, culture and climate.

The diverse South African population is particularly suitable for molecular diagnosis of FH, since specific founder-related LDLR gene defects underlie the disease phenotype in a large proportion of the genetically distinct Afrikaner, Coloured, Jewish, Indian and Black population groups (Henderson et al. 1988, Graadt van Roggen et al. 1991, Kotze et al. 1991, 1995a, Meiner et al. 1991, Rubinsztein et al. 1992, Loubser et al. 1999, Thiart et al. 2000).

6.1 Analysis of the genetically distinct populations of South Africa

An efficient molecular diagnostic strategy depends on the composition of common and rare molecular events (Jensen et al. 1999) and the genetic constitution of the relevant population (Botha and Pritchard 1972). The South African population constitutes at least five genetically distinct populations and a group of immigrants, residing in South Africa for a few decades. The genetics of the Afrikaans-speaking and English-speaking white populations do not differ significantly from one another, but both groups exhibit

Table 1.1 Populations where FH is prevalent due to founder LDLR gene mutations.

Population	Prevalence	Mutation	Allele designation	Reference
SOUTH AFRICA				
Afrikaners	1 in 70	D206E	FH Afrikaner-1	Leitersdorf et al. 1989, Kotze et al. 1989b, 1991
		V408M	FH Afrikaner-2	Leitersdorf et al. 1989, Kotze et al. 1989b, 1991
		D154N	FH Afrikaner-3	Kotze et al. 1989b, 1991
Jews	1 in 100	652delGGT	FH Lithuanian	Meiner et al. 1991
Indians	1 in 100	P664L	FH Gujerat	Kotze et al. 1997
CANADA				
French-Canadians (Quebec-Province)	1 in 270	>Del 15-kb	FH French Canadian-1	Hobbs et al. 1987
		Del 5-kb	FH French Canadian-5	Ma et al. 1989
		W66G	FH French Canadian-4	Leitersdorf et al. 1990
		E207K	FH French Canadian-3	Leitersdorf et al. 1990
		C646Y	FH French Canadian-2	Leitersdorf et al. 1990
		Y468X	-	Simard et al. 1994
FINLAND				
Finnish (North-Karelia)	1 in 441	Del 9-kb	FH Helsinki	Koivisto et al. 1992b
		925del7	FH North Karelia	Aalto-Setälä et al. 1989
		G823D	FH Turku	Koivisto et al. 1995
		L380H	FH Pori	Koivisto et al. 1995
ISRAEL				
Christian Lebanese	1 in 100	C660X	FH Lebanese	Lehrman et al. 1987
Ashkenazi Jews	1 in 80	652delGGT	FH Lithuanian	Meiner et al. 1991
Druze		Y167X	-	Landsberger et al. 1992
Sephardic Jews		D147H	-	Leitersdorf et al. 1993

statistically significant differences from Western European populations with respect to two major blood group systems (Botha and Pritchard 1972). Combined evidence of blood group studies and historical records suggests that the Afrikaans-speaking group possess a measurable genetic contribution of the old Dutch East Indies. The Cape Coloured population group, when subdivided on the basis of religious faith, differs fundamentally in their blood group gene frequencies (Botha and Pritchard 1972).

In clinical practice, a strategy for the genetic diagnosis of heterozygous FH, tailored to the mutational spectrum of patients likely to be seen at the particular hospital / region of the country, would be most efficient (Jensen et al. 1999). Although population-based screening strategies are applied in the molecular diagnosis of FH in South Africa (Kotze et al. 1995b), a more cost-effective and appropriate diagnosis may be possible when the genetically distinct population groups of South Africa are divided into sub-groups according to home language and religious background.

6.1.1 The Afrikaner population

The majority of the ~3 million Afrikaners originated from approximately 2000 settlers who emigrated from Holland, Germany and France in the late 17th and early 18th centuries (Botha and Beighton 1983). The introduction of permanent immigrants was a continuing process with a lesser increment from England in the 19th century, while minor contributions were made from Scandinavia, Eastern Europe, South East Asia and the Mediterranean countries. The indigenous Khoisan people and African Negro slaves, mainly from Madagascar and Mosambique, also contributed to the unique gene pool of the Afrikaner (Botha and Beighton 1983). Approximately 1 million Afrikaners who were founded by one shipload of immigrants in 1652 still have the surnames of 20 original settlers (http://helix.biology.mcmaster.ca/3j3/3j3.fouder). It can therefore be expected that this population would have higher or lower frequencies of certain disease-related alleles, in comparison with their parent European populations. This culturally distinct people remained genetically isolated, reinforced by their religion and language, while undergoing great numerical expansion. In 1867 the ethnic composition of the Afrikaner was constituted of approximately 34.8% Dutch, 33.7% German, 13.2% French, 6.9% Coloured, 5.2% British and 2.7% other nationalities, while 3.5% were unknown (Botha and Beighton 1983).

Three point mutations in the LDLR gene, FH Afrikaner-1 (C to G transversion at codon 206; D206E), FH Afrikaner-2 (G to A transition at codon 408; V408M) and FH

Afrikaner-3 (G to A transition at codon 154; D154N), account for approximately 90% of all clinical FH cases in the Afrikaner population. Mutations, D154N and D206E, residing in exon 4 of the LDLR gene, underlie the FH phenotype in approximately 10% and 65% of Afrikaner FH patients, respectively (Graadt van Roggen et al. 1991, Kotze et al. 1991). These defective alleles are likely to have been introduced into the South African population by British immigrants around 1820 (Botha and Beighton 1983). Both molecular lesions were identified in British FH patients from the Netherlands (Defesche et al. 1993), while mutation D206E was also detected in FH patients from London (Gudnason et al. 1993). Mutation V408M, which originated in the Netherlands (Defesche and Lansberg 1993, Defesche et al. 1993), is responsible for FH in 20-30% of the Afrikaner population (Graadt van Roggen et al. 1991, Kotze et al. 1991) and was also detected in patients from Germany (Schuster et al. 1993). These findings reflect the origin of the Afrikaner.

The Afrikaner FH-1 and FH-3 mutant alleles result in a receptor-defective phenotype, whereas the phenotypic expression of the Afrikaner FH-2 mutant allele corresponds to a receptor-negative phenotype. The FH-1 mutant LDL receptor displays retarded transport (Fourie et al. 1988, 1992), which results in about 20% of the normal LDLR activity. The defective FH-2 allele causes the receptor to be rapidly degraded leading to less than 2% of normal receptor activity (Fourie et al. 1988, 1992). Mutant FH-3 receptors show impaired ability to bind LDL and are poorly expressed on the cell surface as a result of significant degradation of receptor precursors (~20% receptor activity). Patients heterozygous for the FH-1 and FH-3 mutations present with lower plasma cholesterol levels and milder clinical symptoms than those affected with the FH-2 mutation (Graadt van Roggen et al. 1995).

FH appears to be most prevalent in Afrikaners residing in the Witwatersrand region, where the FH phenotype appears to cluster among members of the Gereformeerde Church (Torrington et al. 1984). In the predominantly Afrikaans-speaking community of the Western Cape, of whom the majority belong to religious groups other than the Gereformeerde Church, a high frequency of FH was also noted (Rossouw et al. 1985). Kotze and colleagues (1991) proposed that the relative distribution of the three Afrikaner founder-related mutations may differ in the various geographical regions within South Africa. In a comparative population study on Afrikaner familial hypercholesterolaemics from two distinct regions in South Africa, the Witwatersrand and Western Cape, no differences in mutation frequency could be observed (Graadt van Roggen et al. 1991).

6.1.2 The South African Coloured population

The origins of the Coloured population can be traced back to 1652, to the shores of Table Bay. Marriages between European colonists and the two indigenous populations, the Khoi and the San, was encouraged and the offspring became members of the newly emerging Coloured community. The non-Caucasoid element, apparently including Khoi, San, and Negroid, was not necessarily uniquely African. An additional gene flow to the Coloured gene pool came from the Indian traders, mainly from the Gujerat province, who came to South Africa under British auspices. The genetic contribution from slaves that came from East Africa and Madagascar, who were either completely Negroid or Negroid with Malay or Indonesian admixture, was relatively small. Ancestors of the so-called Cape Malays were largely from the Malay peninsula and islands, including Indonesia (Nurse et al. 1985).

Mutation analysis of the LDLR gene in the Coloured population contributed to our knowledge of the biological history of this unique population and illustrated the potential consequences of recent admixture in populations with different disease risk (Loubser et al. 1999). The detection of multiple founder-type "South African mutations", provided direct genetic evidence that Caucasoid admixture contributed significantly (~20%) to the FH phenotype in South Africans of mixed ancestry. These mutations have most likely been introduced from European (D154N, D206E, V408M), Indian (P664L) and Jewish (652delGGT) populations. The limited data from screening only a portion of the LDLR gene for common disease-causing mutations were consistent with a previous estimation, indicating that the Caucasoid contribution to the gene pool of the Coloured population was approximately 33% (Jenkins 1978, Loubser et al. 1999).

6.1.3 The South African Black population

The South African Black population could be the latest or the most ancient of the major races of man to have emerged (Nurse et al. 1985). This population constitutes three main groups, separated mainly by language (Nurse et al. 1985, Beighton and Botha 1986). Beighton and Botha (1986) acknowledged the Nguni-speaking group, comprising the Zulu, Xhosa and Swazi people, the Northern and Southern Sothos and the Tswana. Minor tribes including the Shangaan-Tsonga, Ndebele and Venda were also mentioned. Nurse and colleagues (1985) divided the Black peoples into three principal groups, the Sotho / Tswana, the Nguni and the Tsonga. During 1980

approximately 17 million black people were living in the area south of the Orange and Limpopo Rivers. The migration of the Black population to South Africa according to history remained controversial, but they may have migrated eastwards from the Congo basin and then southwards into southern Africa. The main arrival at the northern border of South Africa took place nearly 1 000 years ago. By the turn of the fifteenth century (1498) a dense Black population settled at the Limpopo River and by 1552 they apparently established new communities at the eastern Cape coast. By 1752 they migrated, mainly as pastoralists to Natal, the Transvaal and along the eastern coastal region of the Cape Province as far as the Keiskama River. Along their route they partially displaced and absorbed the pre-existing San population, while a degree of hybridisation with the indigenous Khoi occurred. Modernised agricultural, industrial and transport practice led to detribalisation of a large part of the population and their distribution throughout the country. By 1980 nearly half of the population (6.5 million) were urbanised (Beighton and Botha 1986).

The high prevalence of FH in the South African Caucasian population is in striking contrast to its reported virtual absence in the Black population in general. In a study performed by Thiart et al. (2000), predominance of a 6-bp deletion in exon 2 (Leitersdorf et al. 1988) of the LDLR gene was reported in approximately 28% (5/18) of the small number of FH patients that could be recruited for analysis. Detection of this mutation on different haplotypes in several Black groups (Pedi, Tswana and Xhosa), and the fact that the deletion has not been detected in other populations, largely exclude the likelihood of a recurrent mutational event due to slipped mispairing or multiple entries into the Black population. It seems more likely that the 6-bp deletion originated in Africa approximately 3000 years ago before tribal separation.

6.1.4 The South African Indian population

The estimated one million South African Indians are almost entirely descended from 150 000 Indian immigrants who settled in South Africa between 1860 and 1911. The majority (~ 80%) of these immigrants were indentured labourers and came from diverse regions on the south and east coasts of India. A minor group (~ 30 000), the so-called "Passenger-Indians", paid their own passage to South Africa, were traders and artisans and mainly originated from the Gujerat province in Western India. More than half of this group came from the Surat and Valsad districts, while another major source of passenger Indians was Kathiowar on the Arabian Sea (Bhana and Bain 1990).

Although Muslim Gujeratis, until very recently, only married descendants from their own village or locality in India and were genetically isolated in India and abroad (Lazarus 1972), the South African Indian population is of diverse cultural and religious origin (Hindus – 65%, Muslims – 21% and Christians – 7.5%) (Rubinsztein et al. 1994).

The LDLR gene defect, P664L, is most prevalent in South African Indians originating from the Gujerat province of West India (Rubinsztein et al. 1992), and is responsible for the FH phenotype in approximately 50% of affected Indian patients (Kotze et al. 1997). Given the large initial group of Indians that came to South Africa over a period of only three or four generations, it is highly unlikely that a founder effect would have been manifested locally. It is therefore more likely that mutation P664L originated in and was subjected to earlier expansion in India. The relatively high frequency of CpG hotspot mutations in South African Indians with FH may be due to new mutational events and multiple entries of specific LDLR gene mutations into this community.

6.1.5 The South African Jewish population

The Jewish community of Lithuania dates back to 1388 (Ankori 1979). The Ashkenazi Jews are a genetically homogeneous group, living in cultural, environmental, religious and social isolation from the other European populations until the turn of the nineteenth century. A large emigrating process took place and many of them went to South Africa, Great Britain, Latin America, North America and Palestine (Gar 1971). The South African Jewry (Ashkenazi) descended almost exclusively from the north-eastern and central parts of Europe (Lane et al. 1985). The majority of this genetically distinct population group is descendant from an estimated 40 000 Lithuanian immigrants who settled in South Africa between 1880 and 1910, mostly in family groups (Seftel et al. 1989, Meiner et al. 1991, Rubinsztein et al. 1994). The present South African Jewish population is almost totally Ashkenazi (approximately 0.11 million), with more than 60% residing in the Gauteng region (Lane et al. 1985).

The increased prevalence of FH in the South African Jews (1/67) (Seftel et al. 1989) appears to be a consequence of recurrent introduction of specific LDLR gene mutations into these relatively isolated communities. A 3-bp in-frame deletion of Gly197 (652delGGT) (Meiner et al. 1991) underlie the FH phenotype in the majority (80%) of South African Jews. Mutation FH-Lithuania, earlier designated as FH-Piscataway, is

classified as an impaired transport and processing molecular defect, and resulted in extremely low expression of functional receptors at the cell surface (Meiner et al. 1991).

7. OBJECTIVES OF THE STUDY

In the present study a population-directed screening strategy was initially applied to identify common founder-related mutations in the diverse South African population. A combined heteroduplex-single strand conformation polymorphism (HEX-SSCP) technique (Kotze et al. 1995b, Appendix 2), using exon-specific primers (Jensen et al. 1996), was subsequently used to screen for unknown mutations in the promoter and coding regions of the entire LDLR gene. A second screening method, denaturing gradient gel electrophoresis (DGGE) (Nissen et al. 1996), was usually applied when no molecular lesion was detected by HEX-SSCP. PCR-products showing mobility shifts on the appropriate gel systems (Kotze et al. 1995b, Appendix 2) was further analysed by direct DNA sequencing. Either manual sequencing (T7 Sequenase Version 2.0 kit, USB) on 6% polyacrylamide gels, or automated fluorescent sequencing (Big Dye Terminator Cycle Sequencing kit, Perkin Elmer) on an ABI 310 system was performed to identify mutations.

Extensive DNA analysis was performed in South African patients diagnosed with FH, in order to:

- investigate the molecular basis of FH in the diverse South African population
- identify disease-causing mutations in the LDLR gene in hypercholesterolaemic index patients and subsequently in their relatives as part of the MED-PED initiative
- investigate the geographical distribution of the three Afrikaner founder-related mutations in South Africa
- perform genotype-phenotype correlation studies
- assess the contribution of other modifier loci in the phenotypic expression of FH
- contribute to the establishment of a comprehensive predictive diagnostic service for FH in South Africa

CHAPTER 2

Mutation analysis in familial hypercholesterolaemia patients of different ancestries: identification of three novel LDLR gene mutations

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ABSTRACT

Twelve familial hypercholesterolaemia (FH) patients of different ancestries living in South Africa were subjected to mutation analysis of the low density lipoprotein receptor (LDLR) gene. Nine different mutations were identified in 10 patients. Six of these, including the founder-related mutation C660X identified in two Lebanese patients, have previously been described in other FH patients with compatible genetic backgrounds, and/or in patients originating from countries where admixture is not uncommon. Characterisation of an abnormal electrophoresis pattern detected in exon 4 of the LDLR gene by heteroduplex-single-strand conformation polymorphism (HEX-SSCP) analysis, revealed a novel G deletion at codon 185 (617delG) which resulted in a downstream stop codon. Two of the new mutations identified resulted in amino acid substitutions and were designated R57C and Q357P.

KEYWORDS: familial hypercholesterolaemia, low density lipoprotein receptor, mutation screening, polymerase chain reaction

INTRODUCTION

Familial hypercholesterolemia (FH) is a common (1:500) autosomal co-dominantly inherited lipid disorder caused by mutations in the low density lipoprotein receptor (LDLR) gene (Goldstein et al. 1995). Phenotypic variation in heterozygous FH complicates diagnosis. Therefore, characterisation of disease-causing mutations is important for accurate diagnosis, genetic counselling and treatment of this debilitating disease.

In this study nine different LDLR gene mutations were identified in 10 South African FH patients, mostly immigrants from different countries.

MATERIALS AND METHODS

The study population consisted of 12 patients with a clinical diagnosis of FH (Goldstein et al. 1995), attending lipid clinics in South Africa. Blood samples were drawn after obtaining informed consent from patients and approval by the regional ethics review committees. Prior to screening for LDLR gene mutations using restriction enzyme and/or heteroduplex-single-strand conformation polymorphism (HEX-SSCP) analysis (Kotze et al. 1995b, Thiart et al. 1998), familial defective apolipoprotein B-100 (FDB) (Soria et al. 1989) was excluded in all the participants (Kotze et al. 1994). Genomic DNA was amplified by the polymerase chain reaction (PCR), using 20 sets of oligonucleotide primers (Jensen et al. 1996), and PCR products showing aberrant electrophoresis patterns were sequenced directly. Restriction enzyme analyses of exon 14 PCR products in two Lebanese patients were performed by HinfI digestion as previously described (Lehrman et al. 1987).

RESULTS AND DISCUSSION

HinfI digestion of exon 14 PCR products in two Lebanese patients (JD and FP) showed that both are heterozygous for a common founder-related Stop 660 mutation (data not shown). Subsequently, HEX-SSCP analysis of the entire coding and promoter region of the LDLR gene, in the 10 remaining patients, resulted in the identification of different mutations in eight cases (Table 2.1). Three of these mutations, R57C, 617delG and Q357P, were novel and are shown in figure 2.1. The other five mutations, D283N

Table 2.1 LDLR gene mutations identified in FH patients

Patient	Location	Mutation Designation	Nucleotide change	Ethnic background	Reference
KS	exon 3	R57C	C→T at 232	St Helena, Indian, French	This study
JP	exon 4	617delG	DelG at 617	French	This study
JK	exon 4	D206E	C→G at 681	Italian	Kotze et al. 1990
HM	exon 6	D283N	G→A at 910	St Helena, Irish	Bilheimer et al. 1985
AS	exon 8	Q357P	A→C at 1 133	UK	This study
LH	exon 9	W422C	G→C at 1 329	UK	Hobbs et al. 1992
YD	exon 11	G528D	G→A at 1 646	Greek	Hobbs et al. 1992
CJ	exon 14	2092delT	DelT at 2 092	Greek, French	Hobbs et al. 1992
JD	exon 14	C660X	C→A at 2 043	Lebanese	Lehrman et al. 1987
FP	exon 14	C660X	C→A at 2 043	Lebanese	Lehrman et al. 1987

LDLR, low density lipoprotein receptor; FH, familial hypercholesterolemia; del, deletion
mutation names are given according to Beaudet et al. (1996) and Antonarakis et al. (1998)

(Bilheimer et al. 1985), D206E (Kotze et al. 1990), W422C (Hobbs et al. 1992), G528D (Hobbs et al. 1992) and 2092delT (Hobbs et al. 1992), have been described previously. Recurrence of these mutations probably reflects a common origin, since the South Africans/immigrants sharing a specific mutation with another previously characterized FH patient have similar genetic backgrounds or originated from regions where admixture is not uncommon.

The two novel missense mutations, R57C and Q357P, identified in exons 3 and 8 of the LDLR gene, respectively, are expected to cause the FH phenotype in patients KS and AS, since no other disease-causing mutations have been detected by HEX-SSCP and/or sequence analysis of the other gene exons, the promoter region or the splice junctions in DNA samples of these patients. Exons 2 to 6 encode the ligand-binding domain of the receptor molecule and since this region is essential for binding of apolipoprotein (apo) B-100 and apo E to the receptor, mutation R57C is likely to cause a binding- or transport-defective allele (Hobbs et al. 1992). Mutation R57C has furthermore occurred on an evolutionary conserved residue (Mehta et al. 1991). Exons 7 to 14 encode the epidermal growth factor (EGF) precursor homology domain that mediates acid-dependent dissociation of the receptor and ligand in the endosome (Hobbs et al. 1992). It is therefore proposed that mutation Q357P in exon 8 may prevent receptor recycling. Family members were not available to demonstrate co-segregation of the two missense mutations with hypercholesterolemia in the relevant families, but both mutations R57C and Q357P were absent in 100 control chromosomes screened.

The mutation identified in patient JP in exon 4 involves a deletion of a single guanine base after nucleotide 616 at codon 185. This frameshift mutation results in a premature termination codon at amino acid 246 and is likely to be pathogenic, since a truncated protein would be translated.

The results add yet another novel mutation to the relatively large number of sequence variations in exon 4 of the LDLR gene (Hobbs et al. 1992, Varret et al. 1998). The 3'-segment of exon 4 appears to be particularly susceptible to different types of mutational events and therefore represents a mutational hotspot of the LDLR gene (Gudnason et al. 1993, Kotze et al. 1996). This is undoubtedly caused by the sequence characteristics of the specific gene region, that appears to influence the stability of the gene (Gudnason et al. 1993, Peeters et al. 1995).

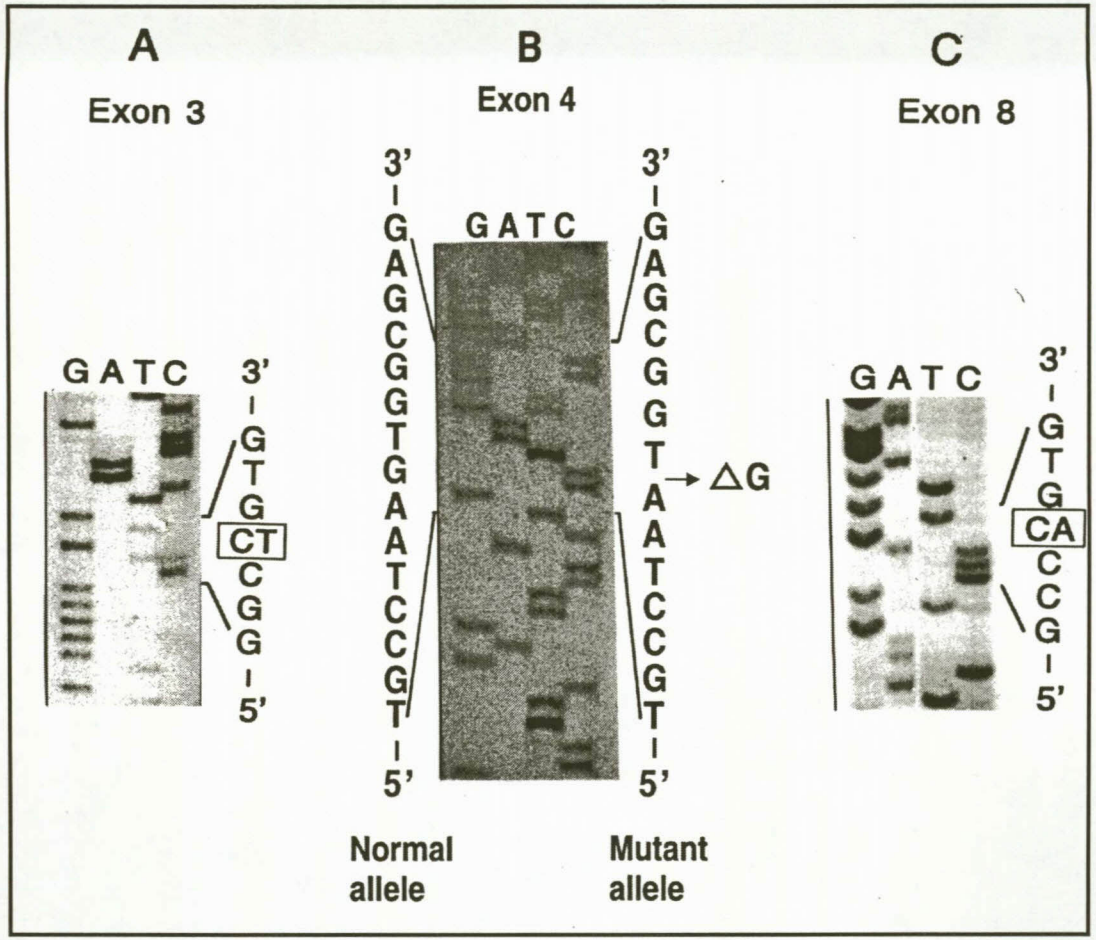

Fig. 2.1 Characterisation of the three novel mutations by sequencing of polymerase chain reaction amplified products: **A** sequence analysis of exon 3 in patient KS; **B** sequence analysis of the mutation in exon 4. The 1-bp deletion is indicated by Δ. Left, sequence of the normal allele; and right, sequence of the mutant allele; **C** sequence analysis of the exon 8 mutation in patient AS.

Failure to detect mutations in two of the samples screened by HEX-SSCP analysis may reflect the inherent limitations of this detection method. Alternatively it may also be an indication that the FH phenotype in molecularly uncharacterized patients is caused by large rearrangements or mutations in another gene(s).

This study demonstrates the importance of a population-directed DNA screening strategy for FH diagnosis. Knowledge that a specific mutation predominates in a specified population group facilitated rapid molecular characterisation in two of the index cases and several of their family members. Mutation analysis of patients from heterogeneous populations, on the other hand, involves a more generalised approach of screening the entire LDLR gene.

ACKNOWLEDGEMENTS

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CHAPTER 3

Familial hypercholesterolaemia: Prevalence of three founder-related LDL receptor gene mutations in the Free State and Northern Cape in comparison with three other geographical regions in South Africa

Submitted to South African Medical Journal

ABSTRACT

Objective. The mutant allele frequencies of three founder-related low density lipoprotein receptor (LDLR) gene mutations causing familial hypercholesterolaemia (FH) in approximately 90% of Afrikaners were determined in the Free State and Northern Cape, for comparison with the frequencies reported in three other distinct geographical areas within South Africa.

Methods. Population-directed molecular diagnosis of FH was performed in 474 Caucasians of Afrikaner lineage, using a multiplex polymerase chain reaction (PCR)-based assay to simultaneously screen for LDLR gene mutations D154N, D206E and V408M.

Results. The distribution of the three Afrikaner mutations detected in the Free State and Northern Cape was in general agreement with that described previously in the Western Cape, Gauteng and North-West provinces. The most prevalent mutation in all four geographical regions was D206E (63% combined average), followed by V408M (31.4%) and D154N (5.6%). The frequency of mutation V408M was significantly increased ($P<0.05$) in the Free State, Northern Cape and North-West regions, with a concurrent decreased frequency of mutation D206E, compared to previous estimates for the Western Cape and Gauteng.

Conclusions. The relative frequencies of the three Afrikaner founder-related mutations in different regions in South Africa largely correlate with the distribution of home language and historical affiliation with the Gereformeerde Church.

KEYWORDS: familial hypercholesterolaemia, founder-related mutations, geographical distribution

INTRODUCTION

Familial hypercholesterolaemia (FH) is an autosomal co-dominant disease caused by mutations in the gene encoding the low density lipoprotein receptor (LDLR), which promotes the clearance of cholesterol-rich LDL particles from blood. More than 600 different LDLR gene mutations causing FH have been reported in different European populations (http://www.ucl.ac.uk/fh; http://www.umd.necker.fr) (Day et al. 1997, Varret et al. 1998), with an estimated prevalence of 1 in 500 for heterozygotes and 1 in a million for homozygotes (Goldstein et al. 1995).

In the South African Afrikaans-speaking whites (Afrikaners) (Kotze et al. 1991), French-Canadians (Leitersdorf et al. 1990), Christian Lebanese (Lehrman et al. 1987), Finns (Aalto-Setälä 1988) and the Ashkenazi Jews of Lithuanian ancestry (Meiner et al. 1991), founder effects have resulted in an extremely high prevalence of specific FH-related mutations. The founder mutations most likely originated as a result of the relative genetic isolation of these groups through geography, language, religion and culture, while undergoing great numerical expansion. Among Afrikaners the frequency of this disease has been increased to approximately 1 in 70 individuals (Jooste et al. 1986, Steyn et al. 1996), rendering FH the most lethal inherited disorder in this population.

From the estimated 10 million people affected with FH worldwide, the vast majority are unaware of their significantly increased risk for the development of coronary heart disease (CHD). According to the South African co-ordinators of an international initiative aimed at "Making Early Diagnosis to Prevent Early Deaths in MEDical PEDigrees with Familial Hypercholesterolaemia" (MED-PED FH project), approximately 80 000 FH heterozygous South Africans have not yet been identified and of the expected 200 homozygotes, less than 100 have been diagnosed with FH (Kotze et al. 1998a). Afrikaners exhibit one of the highest death rates from CHD in the Western world, mainly in the economically active period of their lives (Wyndham 1978, Pretorius 1983). In this population group, the mortality rate in men aged 25 to 35 years is twice as high as reported in North America, more than three times higher than in Finland and more than seven times higher than in Sweden (Wyndham 1978). It is estimated that males diagnosed with FH have up to a 40-fold increased risk of premature CHD and if untreated, their life expectancy is reduced by 20 to 30 years. If left untreated, males usually die of CHD between the ages 35 to 55, and women between 55 and 75 years of age (Williams et al. 1993b).

Three point mutations in the LDLR gene, FH Afrikaner-1 (D206E), -2 (V408M) and -3 (D154N), account for approximately 90% of all clinical cases of FH in the Afrikaner population, and are also prevalent in South Africans of mixed ancestry (Cape Coloureds) due to Caucasoid admixture (Loubser et al. 1999, Kotze et al. 1995a). The FH-1 and FH-3 mutations result in a receptor-defective phenotype (~20% of normal receptor activity), whereas the phenotypic expression of the Afrikaner FH-2 mutant allele corresponds to a receptor-negative allele (<2% activity) (Fourie et al. 1988, Graadt van Roggen et al. 1995).

Mutation analysis previously performed in the Western Cape (Kotze et al. 1992), Gauteng (Graadt van Roggen et al. 1991) and North-West provinces (Oosthuizen et al. 1995) demonstrated that the Afrikaner FH-1 mutation was most prevalent in all these regions, followed by the FH-2 mutation. The mutant FH-2 allele frequency of the North-West region showed a marked increase compared to the other two regions. Although the mutant FH-3 allele was far less prevalent than genotypes FH-1 and FH-2, the frequency of this mutation differs greatly between these three areas. Western Cape exhibits an allele frequency of 11.3% for the Groote Schuur Hospital sampling point and 10.3% for the Tygerberg Hospital sampling depot. In contrast, the FH-3 allele frequency was calculated at 3.4% for the Gauteng region and appeared to be completely absent in the North-West region. These results were mainly based on white Afrikaans-speaking patients attending lipid disorder clinics at Potchefstroom (Oosthuizen et al. 1995) (representing the North-West Province), Johannesburg (Graadt van Roggen et al. 1991) (representing Gauteng), Tygerberg (Kotze et al. 1991, 1992) Groote Schuur (Graadt van Roggen et al. 1991) and Red Cross War Memorial Children's (Graadt van Roggen et al. 1991) (representing the Western Cape) hospitals (Fig. 3.1). In the present investigation we extended the sampling depots to include four distinct geographical areas within South Africa and compared the mutant allele frequencies of the founder-related Afrikaner FH-1, FH-2 and FH-3 mutations.

MATERIALS AND METHODS

Patients and reference criteria

Over a period of approximately five years, a total of 474 apparently unrelated subjects were screened for the presence of the three founder-related LDLR gene mutations, FH-1 (D206E), -2 (V408M), and -3 (D154N). Of these, 244 hypercholesterolaemic index patients were referred for a molecular diagnosis of FH via the lipid clinic at Universitas

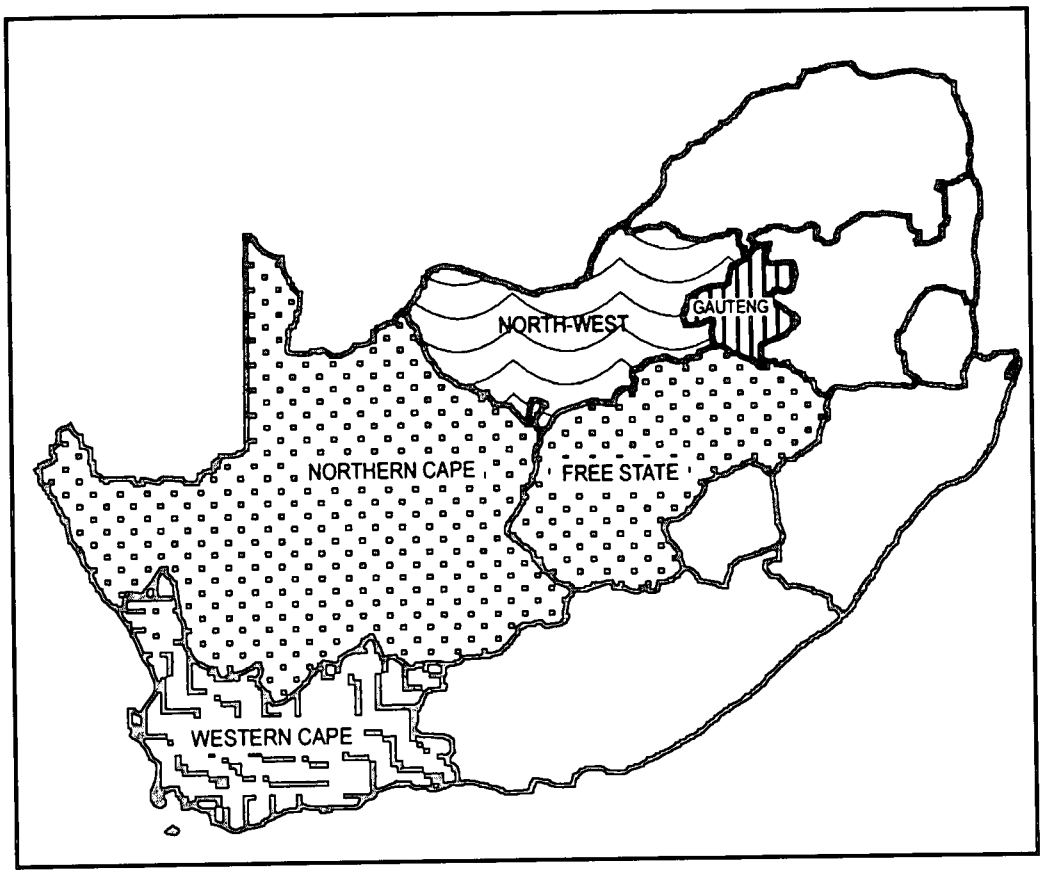

Fig. 3.1 The different regions in South Africa included for comparative analysis of LDLR gene mutation frequencies. The lipid disorders clinic at Potchefstroom serves the North-West Province, the Johannesburg Hospital lipid clinic the Gauteng area, the lipid clinics at the Red Cross War Memorial Children's Hospital, Tygerberg Hospital, and Groote Schuur Hospital serve the Western Cape area, and the Universitas Hospital lipid clinic the Free State and Northern Cape regions.

hospital, Bloemfontein, as well as pathology firms in the Free State and Northern Cape. The clinical diagnosis of FH was based on the following criteria: (i) total serum cholesterol (TC) levels ≥ the 80th percentile for age and gender (if patients received cholesterol-lowering drugs, they were included when their TC levels were at least equal to the 70th percentile for age and gender), (ii) serum triglyceride levels < 2.3 mmol/l, and (iii) the presence of tendon xanthomata and/or a family history of early CHD (Rossouw et al. 1985). A family history of early CHD was defined as being present when the patient reported that at least two first-or second-degree relatives suffered fromCHD before the age of 55 years. Although most referrals met the above-mentioned criteria, some of the index patients were referred for a molecular diagnosis of FH solely

on the basis of raised TC levels. Follow-up mutation screening was subsequently performed in 185 family members of 120 mutation-positive cases.

Mutation screening strategy

Genomic DNA was extracted from freshly-drawn or frozen peripheral blood using standard procedures (Kotze et al. 1987). The DNA samples were analysed using a nonradioactive multiplex amplification refractory mutation system polymerase chain reaction (ARMS-PCR) screening strategy developed by Kotze et al. (1995b). Patient samples were also screened for the common apolipoprotein (apo) B3500 mutation causing familial defective apolipoprotein B-100 (FDB) (Soria et al. 1989), by including an allele-specific primer set (Wenham et al. 1991) in the multiplex assay.

Statistical analysis

Chi square (χ^2) values were calculated and their significance levels determined by two-way contingency tables. P values <0.05 were regarded as statistically significant.

RESULTS AND DISCUSSION

A population-directed molecular diagnosis of FH focusing on the three founder LDLR gene defects was performed in 474 Caucasians of Afrikaner lineage, resident in the Free State and Northern Cape regions of South Africa (Table 3.1). Of the 474 patients analysed, 208 (43.9%) tested positive for one of the three founder-related Afrikaner mutations. One hundred and ninety nine patients were heterozygous, one was a homoallelic FH homozygote (FH-1/FH-1) and eight were compound heterozygotes (7 FH-1/FH-2 and 1 FH-1/FH-3). The FH-1 mutation was detected in 115 (55.3%) FH heterozygotes, FH-2 in 78 (37.5%) and FH-3 in six (2.9%) patients. Follow-up mutation screening conducted in 185 family members of 120 mutation-positive index patients demonstrated a defective LDLR gene in almost half (89 subjects) of these siblings, as expected for an autosomal dominant genetic disorder. None of the families analysed tested positive for the apo B3500 mutation causing FDB, which is clinically indistinguishable from FH.

Table 3.1 Distribution of the three founder-related Afrikaner LDLR gene mutations in the Free State and Northern Cape

	FH mutant alleles*						Unknown Defects
	FH-1/N	FH-2/N	FH-3/N	FH-1/FH-1	FH-1/FH-2	FH-1/FH-3	
FH patients (n=474)	115	78	6	1	7	1	266
	55.3%	37.5%	2.9%	0.5%	3.4%	0.5%	

Total number of mutation-positive FH patients: 208 (43.9%)

*Percentages were calculated as a percentage of the total number of mutation-positive patients

The distribution of the three Afrikaner founder mutations detected in the Free State and Northern Cape was in general agreement with that described previously in the Western Cape, Gauteng and North-West provinces (Table 3.2). The FH-1 mutation was most prevalent in all four geographical regions, at a combined average of 63.0%, compared to 31.4% and 5.6% for the FH-2 and FH-3 mutations, respectively. It has previously been suggested that the low frequency of the FH-3 mutation in South Africa can probably be explained by the fact that the founder immigrant who introduced this defective gene into the South African population had only three children, of whom only one presumably transmitted the defective gene to successive generations (Torrington and Brink 1990).

In a previous study of Afrikaners (Kotze et al. 1991), it was suggested that the proposed association between FH and affiliation to the Gereformeerde Church (Torrington et al. 1984), a minor South African church denomination, may be reflected by differences in relative frequencies of the founder mutations in different parts of South Africa. However, this hypothesis was not supported by the data presented by other investigators (Graadt van Roggen et al. 1991), reporting a similar distribution of the mutations in the Western Cape and Gauteng regions. Notably the FH-3 mutation,

Table 3.2 Frequencies of the founder-related FH mutant alleles in South African patients from different geographical regions

Geographical Regions	FH mutant alleles					
	FH-1		FH-2		FH-3	
	Number of alleles	%	Number of alleles	%	Number of alleles	%
Free State and Northern Cape (n=217)	125	57.6	85	39.2	7	3.2
North-West [a] (n=107)	61	57.0	46	43.0	0	0.0
Western Cape						
Groote Schuur and Red Cross War Memorial Children's Hospitals [b] (n=53)	34	64.2	13	24.5	6	11.3
Tygerberg Hospital [c] (n=107)	73	68.2	23	21.5	11	10.3
Gauteng [b] (n=59)	40	67.8	17	28.8	2	3.4
Average (%)		**63.0%**		**31.4%**		**5.6%**

P values were determined for mutation FH-2 in the different regions:
Free State and Northern Cape vs Groote Schuur and Red Cross War Memorial Children's Hospitals: P=0.047; Free State and Northern Cape vs Tygerberg Hospital: P=0.002; North-West vs Groote Schuur and Red Cross War Memorial Children's Hospitals: P=0.023; North-West vs Tygerberg Hospital: P=0.001; No statistical differences were observed in the other regions.

[a]Data reproduced from Oosthuizen et al. 1995, [b]Graadt van Roggen et al. 1991, [c]Kotze et al. 1992.

showing no correlation with affiliation to the Gereformeerde Church (Torrington and Brink 1990), was shown by both groups of investigators (Graadt van Roggen et al. 1991, Kotze et al. 1991) to have a three times higher frequency in the Western Cape (lowest Gereformeerde Church membership), compared to the other three geographical regions (Table 3.2), where the majority of this church denomination resides (Table 3.3). Results of the present investigation demonstrated a significantly increased ($P<0.05$) frequency of the FH-2 mutation in the Free State, Northern Cape and North-West provinces, compared with the Western Cape area. A concurrent decreased FH-1 mutation frequency was observed in the Western Cape and Gauteng regions.

The FH-2 mutation in exon 9 of the LDLR gene can be traced back to the Netherlands (Defesche et al. 1993), which is also the country of origin of the founder members of the Gereformeerde Church, founded in 1886. This mutation can therefore be considered the most likely candidate of the three Afrikaner founder mutations to be associated with Gereformeerde Church affiliation, particularly since the two exon 4 mutations were also detected in FH patients of English descent in Europe (Defesche and Lansberg 1993, Gudnason et al. 1993). Further support for this view was provided by mutation screening of patients from Potchefstroom and surroundings (North-West Province) (Oosthuizen et al. 1995), where an FH-2 mutant allele frequency of 43% was found, which is the highest known incidence in South Africa for the FH-2 Dutch-associated allele. These data are of particular significance since the Theological School of the Gereformeerde Church has been situated in Potchefstroom since 1904 (De Bruyn 1977), and the province also houses the highest percentage (5.8%) of Gereformeerde Church members (Provincial Statistics 1994d).

Of further interest is the likelihood that the higher FH-2 mutation frequency in the Free State, Northern Cape and the North-West provinces may be a consequence of population distribution regarding home language in the four distinct areas under discussion (Table 3.3). In the Western Cape and Gauteng the percentage of Afrikaans-speaking people are more or less equal, namely 52.6% and 54.7%, respectively. The same trend is observed for the English-speaking section of these two populations (44.1% and 40.2%, respectively). The language distribution between Afrikaans-and English-speaking people in the Free State and Northern Cape, however, differs significantly from the Western Cape and Gauteng provinces (Table 3.3). The

Table 3.3 Distribution of home language and affiliation to the Gereformeerde Church among whites

	Afrikaans (%)		English (%)		Gereformeerde Church (%)	
	1951 [a]	1994 [b]	1951 [a]	1994 [b]	1951 [a]	1994 [b]
Free State and Northern Cape*	84.8	89.0	13.3	9.5	5.9	3.2
North-West	58.9	88.8	36.6	9.0	5.4	5.8
Western Cape	57.8	52.6	39.2	44.1	3.2	0.5
Gauteng	58.9	54.7	36.6	40.2	5.4	2.7

* Average percentage for the two provinces

[a] Data reproduced from Union Statistics for fifty years: 1910-1960 (1960)

[b] Data reproduced from Provincial Statistics (1994a, 1994b, 1994c, 1994d, 1994e)

percentage of Afrikaans-speaking people (89%) in the Free State and Northern Cape is almost 10 times higher than the English-speaking section (9.5%) of the South African Caucasian population. The Afrikaans-speaking group is expected to show a higher prevalence of the Dutch-associated FH-2 mutation, and therefore the higher frequency of this mutation in the Free State, Northern Cape and the North-West Province is in accordance with the distribution of home language.

The proportional distribution of home language in the North-West Province (Afrikaans-speaking residents: 88.8%, English-speaking residents: 9.0%) almost resembles that of the Free State and Northern Cape. In this province the FH-2 Dutch-associated allele is more frequent (43.0%) than in any of the other geographical regions within South Africa (Oosthuizen et al. 1995). The decreased frequency of the FH-1 mutation in the Free Sate and Northern Cape areas where English is spoken by the minority, compared to the Western Cape and Gauteng regions, support this notion. In

accordance, a decrease in both apparently English-associated alleles, FH-1 (57.0%) and FH-3 (0%) was found in the North-West Province (Oosthuizen et al. 1995).

We conclude that the relative frequencies of the three Afrikaner founder-related mutations in different regions in South Africa correlate significantly with the distribution of home language and historical affiliation with the Gereformeerde Church. The widespread distribution of all three LDLR gene mutations in South Africa emphasises the diagnostic value of the multiplex ARMS-PCR assay (Kotze et al. 1995b), developed for simultaneous analysis of all three founder mutations underlying this disease in the vast majority of FH patients in South Africa (Kotze et al. 1991, Loubser et al. 1999). Population-directed screening for known LDLR gene mutations enables accurate diagnosis of FH, a prerequisite for optimal management of this treatable genetic disease. The international MED-PED FH initiative, established to promote tracing of defective genes in extended FH pedigrees, can be considered a paradigm for many other common founder-related genetic diseases in South Africa (Kotze and Callis 1999).

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CHAPTER 4

Mutational spectrum of LDLR gene mutations underlying familial hypercholesterolaemia in South African Jews

ABSTRACT

Objective. A cohort of 21 unrelated index patients with familial hypercholesterolaemia (FH) was screened for mutations in the low density lipoprotein receptor (LDLR) gene, in order to define the spectrum of mutations underlying the FH phenotype in the South African Jewish population.

Methods. Multiplex polymerase chain reaction (PCR) amplification for detection of the prevalent South African founder mutations was applied, followed by heteroduplex-single strand conformation polymorphism (HEX-SSCP) analysis and/or denaturing gradient gel electrophoresis (DGGE) in mutation negative samples, to identify LDLR gene defects in South African Jews with clinical signs of FH.

Results. The FH Lithuania mutation (652delGGT) reported in the majority of South African Jews with FH, was detected in 57% (12/21) of the index cases studied. Seven of the remaining nine index patients were subjected to extensive mutation analysis of the LDLR gene and mutations Q64X, Q104W, D206E, IVS9+1G→A, N407K, V408M and IVS14+5G→A were identified. Mutation N407K is associated with a mild FH phenotype.

Conclusions. Predominance (75%) of the founder-related deletion-mutation, 652delGGT, was confirmed in South African Jewish FH patients of Lithuanian origin. The finding that mutation N407K may be age-dependent, stresses the importance of family follow-up for accurate disease diagnosis, a prerequisite for preventive treatment.

KEYWORDS: familial hypercholesterolaemia, South African Jews, genotype-phenotype correlation

INTRODUCTION

Familial hypercholesterolaemia (FH) contributes significantly to the high mortality rate from coronary heart disease (CHD) in Westernised countries. Fifty percent of men and 20% of women with heterozygous FH usually have clinical evidence of CHD by age 45. This autosomal co-dominantly inherited disease is caused by mutations in the low density lipoprotein receptor (LDLR) gene located on chromosome 19p13.1-p13.3 (Goldstein et al. 1995). To date more than 600 mutations (http://www.ucl.ac.uk/fh and http://umd.necker.fr) underlying FH have been identified (heterozygote frequency of 1/500).

Molecular diagnosis of FH can be applied cost-effectively in genetically homogeneous populations where a limited number of mutations predominate. The South African population is particularly suitable for DNA diagnosis of FH, since relatively few founder-type LDLR gene mutations are responsible for this disease in a large proportion of patients from different ethnic groups (Kotze et al. 1991, 1997, Meiner et al. 1991, Rubinsztein et al. 1992, Loubser et al. 1999, Thiart et al. 2000). The most common mutations, D206E, D154N and V408M, cause FH in more than 90% of affected Afrikaners (Kotze et al. 1991), while a 3-bp in-frame deletion of codon 197 (652delGGT) was reported in 80% of South African Jews with FH (Meiner et al. 1991).

In a survey conducted in the Jewish population resident in Johannesburg and surroundings, an exceedingly high prevalence of FH was reported based on clinical, biochemical and family studies. A heterozygote frequency of 1/67, which is seven times higher than reported in most other countries, was established (Seftel et al. 1989). This high prevalence of FH in the Johannesburg Jewish community has important health implications, particularly since the mortality rate from CHD is reported to be exceedingly high in this group (Walker 1963). The present South African Jewish community (0.11 million) is almost totally of Ashkenazi descent and approximately 60% (66 000) are living in the Johannesburg region (Gauteng province). South African Jews descended almost exclusively from the north-eastern and central parts of Europe (Lane et al. 1985).

Characterisation of FH-causing mutations in the LDLR gene is important for accurate diagnosis of FH. In this study 21 unrelated FH index patients were screened for mutations in the LDLR gene, in order to define the spectrum of mutations underlying the FH phenotype in the South African Jewish population.

MATERIALS AND METHODS

Patients

Blood samples were obtained with informed consent from 21 unrelated South African Jewish patients attending the Johannesburg lipid disorders clinic, as well as eight family members. The clinical diagnosis of FH was based on raised plasma cholesterol levels, the presence of tendon xanthomata and/or a family history of premature coronary heart disease (CHD). Levels of total plasma cholesterol (TC), high density lipoprotein cholesterol (HDLC) and triglycerides (TG) were determined as previously described (Kotze et al. 1987).

Follow-up studies were performed in family members of patient JO, who presented with clinical features suggestive of homozygous FH. This patient (heterozygous for both mutations D206E and N407K) responded well on an expanded-dose of simvastatin (Raal et al. 1997a). Her untreated TC levels were between 12 and 14 mmol/l and onset of CHD was at age 24 years. Tendon xanthomata were detected at the age of 33 years. She underwent coronary angioplasty at age 21 and bypass surgery at ages 24 and 35 years. Her mother passed away at age 63 due to a stroke and her father at age 58 as a result of a myocardial infarction.

DNA analysis

Genomic DNA was extracted using a standard method (Miller et al. 1998). A polymerase chain reaction (PCR)-based screening strategy (Kotze et al. 1995b) was applied to simultaneously screen for the presence of three Afrikaner founder-related mutations (D154N, D206E and V408M) (Kotze et al. 1991) and deletion Gly197 (652delGGT) (Meiner et al. 1991). Combined heteroduplex-single strand conformation polymorphism (HEX-SSCP) analysis (Kotze et al. 1995b) using exon-specific primers (Jensen et al. 1996), and/or denaturing gradient gel electrophoresis (DGGE) (Nissen et al. 1996), was used to screen for unknown mutations in seven of the nine mutation-negative subjects of whom sufficient DNA was available. Direct sequencing on an automated ABI 310 system was subsequently performed to identify mutations in PCR amplified products demonstrating mobility shifts. Known mutations causing familial

defective apolipoprotein B-100 (FDB) (Soria et al. 1989, Kotze et al. 1994) was excluded in the study participants prior to screening for LDLR gene mutations.

RESULTS AND DISCUSSION

Eight different LDLR gene mutations were identified in 21 apparently unrelated South African Jewish FH patients (Table 4.1). Twelve patients were heterozygous for the Jewish founder-related mutation, 652delGGT. Mutation Q64X, previously reported at a relatively low frequency in the German population (Schuster et al. 1995), was detected in two immigrants of Jewish-German descent. One at-risk relative was also shown to be heterozygous for mutation Q64X.

Previous mutation analyses performed in a small cohort of South African Jewish FH patients demonstrated a predominance (80%, 8/10) of mutation 652delGGT (Meiner et al. 1991). This 3-bp deletion was detected in 57% (12/21) of Jewish FH patients included in the current study, of whom nine (75%) were of Lithuanian descent. Three non-Lithuanian Jewish patients were positive for mutation 652delGGT. Mutation IVS14+5G→A (Heath et al. 1999) was identified in one of two Jews of exclusive Lithuanian lineage without the common deletion-mutation.

In an attempt to determine the potential significance of mutation N407K detected in combination with the receptor-defective mutation D206E in a patient (JO) with clinical signs suggestive of homozygous FH, family follow-up studies were performed. Her two sons aged 16 and 20 years, presented with normal TC levels of 4.73 mmol/l and 4.37 mmol/l, respectively, although both were heterozygous for mutation N407K. When the pedigree analysis was extended (Fig. 4.1), and lipid determinations repeated in the eldest son three years later, his TC level was elevated above the 90th percentile for age and gender. It has been noted that children with FH-related mutations may initially present with lipid levels within the normal range, with elevated levels only developing at a later age, but the frequency of this phenomenon is unknown (Kessling et al. 1990).

Both mutations D206E and N407K were identified in the proband's sister (II:2), who presented with a TC level of 7.3 mmol/l. She has apparently responded very well to lipid-lowering drugs, since her pretreatment TC concentration was reported to be approximately 20 mmol/l. Her daughter (III:1) and son (III:2) tested positive for mutations N407K and D206E, respectively, in association with elevated TC levels (>7 mmol/l).

Table 4.1 Spectrum of LDLR gene mutations identified in 21 unrelated South African Jewish FH patients

Exon / Intron	Molecular Event	Designation	No. of patients	References
Exon 3	C→T at 253	Q64X	2	Schuster et al. 1995
Exon 4	C→T at 373	Q104X	1	Bertolini and Callandra 1998
Exon 4	Del GGT at 652	652delGGT	12	Meiner et al. 1991
Exon 4	C→G at 681	D206E	1*	Leitersdorf et al. 1989
Intron 9	G→A at 1358+1	IVS9+1G→A	1	Top et al. 1993
Exon 9	C→G at 1284	N407K	1*	Raal et al.1997a
Exon 9	G→A at 1285	V408M	1	Kotze et al. 1989b
Intron 14	G→A at 2140+5	IVS14+5G→A	1	Heath et al. 1999

* compound heterozygote for mutations D206E and N407K

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

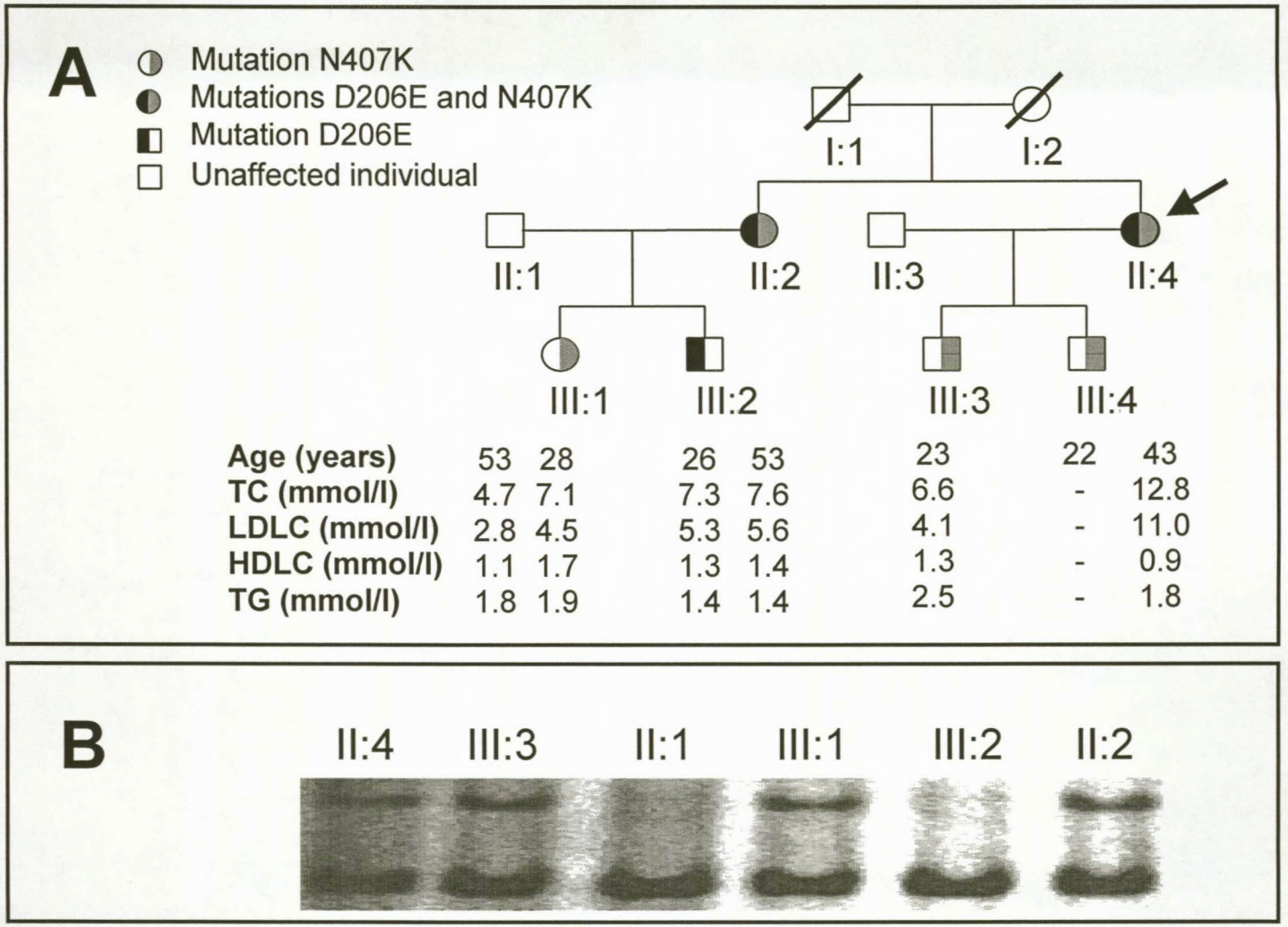

Fig. 4.1 Segregation analysis of mutations D206E and N407K in an Afrikaner-Jewish family. (A) Pedigree structure with ages and lipid profiles indicated (TC, Total cholesterol; LDLC, Low density lipoprotein cholesterol; HDLC, High density lipoprotein cholesterol; TG, Triglyceride). (B) Detection of LDLR gene mutation, N407K applying the HEX-SSCP screening strategy.

Since mutation N407K is located immediately adjacent to the common Afrikaner founder mutation V408M (Kotze et al. 1989b, Leitersdorf et al. 1989) in the highly conserved precursor homology domain of the LDLR gene (Mehta et al. 1991), it might be expected that this mutation would result in a similar recycling-deficient LDLR phenotype with relatively severe clinical consequences (Kotze et al. 1993b). In table 4.2 the phenotype of patient JO with mutations D206E and N407K is compared with that of 13 D206E/V408M hetero-allelic homozygotes. The Afrikaner-Jewish patient with both mutations D206E and N407K was older (39 years) than all the D206E/V408M compound heterozygotes studied (mean age 12.3 ± 7.2 years) and presented with the lowest pretreatment plasma cholesterol levels. At first, these findings raised the possibility that mutation N407K alone does not cause the disease phenotype but may exert some aggravating effect in the presence of another FH-causing mutation, particularly since the carrier offspring initially presented with normal TC levels. It is known that mild types of FH exist and that certain LDLR mutations, when present in the heterozygous state, may cause only a moderate elevation of serum cholesterol (Kajinami et al. 1989, Koivisto et al. 1993). Support for the clinical significance of mutation N407K was obtained only after extended genotype-phenotype correlation studies were performed in the family members of the index patient. This stresses the importance of family follow-up studies for implementation of preventative treatment strategies before the onset of CHD.

This study demonstrates the potential value of mutational screening in a population with a certain enrichment of particular gene mutations and emphasises the need for comprehensive mutation screening and family studies in diagnostic settings. It also demonstrates that combined screening techniques gives a high sensitivity for detecting causative mutations, as mutations were found in all 19 extensively examined samples in this study. The high prevalence of deletion-mutation, 652delGGT, was confirmed in Jewish patients of Lithuanian origin and remains the most common mutation in this relatively homogeneous group. The demonstration that mutational heterogeneity in the LDLR gene may influence the phenotypic expression of FH (Kotze et al. 1993b, Moorjani et al. 1993) has emphasised the need for rapid screening methods that may assist in the assessment of an individual's risk for the development of CHD.

Table 4.2 Comparison of the phenotypic expression of compound heterozygosity for mutations D206E/N407K in patient JO with 13 Afrikaner D206E/V408M FH homozygotes

Patient Designation	Sex	Age (yrs)	TC	LDLC	HDLC	TG	XMTA	CHD
			(mmol/l)					
D206E/N407K								
JO	F	39	12.8	11.1	0.9	1.8	+	+
D206E/N407K								
401	F	6	15.2	14.4	0.6	0.9	+	+
253	F	7	21.8	20.6	0.9	1.4	+	-
867	F	19	24.6	23.3	0.6	1.6	+	-
875	F	10	18.8	18.1	0.4	0.6	+	-
881	F	17	18.1	17.4	0.5	1.0	+	-
890	F	11	19.7	18.5	0.7	1.0	+	+
904	F	1	25.7	24.3	0.5	2.0	+	-
35	F	28	19.2	17.6	1.3	1.3	+	-
642	F	20	18.5	17.6	0.6	0.7	+	+
643	F	10	13.6	12.6	1.2	1.2	+	+
32	F	11	23.9	22.6	0.6	1.7	+	+
33	M	6	23.7	22.2	1.7	1.7	+	+
416	F	14	19.5	17.8	0.8	1.7	+	+
	Mean	**12.308**	**20.177**	**19.000**	**0.800**	**1.292**		
	SD	**7.216**	**3.625**	**3.457**	**0.381**	**0.435**		

Pre-treatment lipid levels are given for total cholesterol (TC), low density lipoprotein cholesterol (LDLC), high density lipoprotein cholesterol (HDL) and triglycerides (TG); tendon xanthomata (XMTA), coronary heart disease (CHD)

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CHAPTER 5

Homozygous familial hypercholesterolaemia: Multiple founder mutations underlie phenotypic variation in the South African population

ABSTRACT

Objective. Predominance of three low density lipoprotein receptor (LDLR) gene mutations in South African Afrikaner patients with familial hypercholesterolaemia (FH) significantly enhances the diagnostic prospects of the disease in this population. The degree of genetic heterogeneity in Afrikaner FH homozygotes was studied in relation to the phenotypic expression of the disease, in order to determine whether additional mutations should be included for routine DNA diagnosis, and to assess whether mutation status may provide useful parameters of disease progression in homozygous FH.

Methods. Polymerase chain reaction (PCR)-based methods were used to screen FH homozygotes without (or with only one copy of) the three founder-related mutations, D154N, D206E and V408M, for disease-related LDLR gene mutations. Afrikaner FH homozygotes with these mutations known to cause a receptor-defective or -negative phenotype, were grouped according to genotype for comparative analysis.

Results. Mutations W66G, D200G (heterozygous state) and S285L (homozygous state) were identified in three of the four Afrikaner FH homozygotes subjected to mutation analysis. Significantly higher mean total- ($P<0.008$) and LDL-cholesterol ($P<0.015$) levels were demonstrated in 24 FH homozygotes with at least one copy of the receptor-negative mutation V408M, compared with 23 patients with receptor-defective LDLR gene mutations D154N and/or D206E.

Conclusions. Mutation analysis provides useful parameters on which to base predictions of clinical progression of FH. Inclusion of all relevant mutations in routine DNA screening would therefore facilitate an improved diagnostic service for FH in the genetically homogeneous Afrikaner population.

KEYWORDS: homozygous familial hypercholesterolaemia, genotype-phenotype correlation, low density lipoprotein receptor, founder mutations

Introduction

Homozygous familial hypercholesterolaemia (FH) is characterised by severe clinical manifestation of tendon xanthomata and atherosclerosis, usually associated with premature death during childhood (Goldstein et al. 1995). 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). A definite diagnosis of FH can be established by the application of genetic testing to trace the defective gene in families (Kotze et al. 1993a, Kotze and Callis 1999).

The mutational heterogeneity of FH appears to explain most of the clinical variation found among FH homozygotes (Moorjani et al. 1993) and may also account for phenotypic variability in heterozygous FH (Kotze et al. 1993b, Gudnason et al. 1994, Pimstone et al. 1998). A strong correlation between residual receptor activity and expression of the clinical manifestations of homozygous FH has already been established prior to association studies involving DNA analysis (Sprecher et al. 1985, Yamamoto et al. 1989). Since the expression of coronary heart disease (CHD) and premature death are related to low density lipoprotein receptor (LDLR) status (Goldstein et al. 1982, Yamamoto et al. 1989), determination of lipid profiles, LDLR activity and the mutations involved may provide useful parameters on which to base predictions of clinical progression of the disease. The occurrence of CHD was reported in 50% of homozygous Afrikaner patients before the age of 9 years (Seftel et al.1980), while 25% of homozygous Japanese patients usually suffer from CHD by age 10 (Mabuchi et al. 1978).

Multiple founder-type LDLR gene mutations contribute to the high death rate from CHD in South Africa. Mutations D154N, D206E and V408M account for FH in approximately 90% of affected cases in the South African Afrikaans-speaking white (Afrikaner) population (Kotze et al. 1989b, 1990, Leitersdorf et al. 1989). These mutations, as well as two LDLR gene mutations underlying FH in the majority of South African Jews (652delGGT) (Meiner et al. 1991, Callis et al. 2001a) and Indians (P664L) (Rubinsztein et al. 1992, Kotze et al. 1997), are responsible for this disease in 15-20% of patients of mixed ancestry (Loubser et al. 1999). Frequent detection (28%) of a 6-bp deletion (137del6) in FH patients from the South African black population, where this disease appears to be extremely rare, suggests that FH may be underdiagnosed in this

group and/or may be associated with a selective advantage within the African context (Thiart et al. 2000).

The most common (~60%) Afrikaner LDLR gene mutation, D206E, results in the formation of two functionally distinct forms of the mutant receptor. One form exhibits normal receptor activity whereas the other is unable to bind lipoprotein ligands (Fourie et al. 1988, 1992). As a result, fully upregulated cells homozygous for this mutation express about 20% of normal receptor activity. Mutation D154N occurring in approximately 10% of Afrikaner FH patients also represents a receptor-defective allele and exhibits ~20% of normal receptor activity. The mutant receptor is characterised by reduced affinity to bind LDL and is poorly expressed on the cell surface due to degradation of the precursor molecule (Graad van Roggen et al. 1995). In contrast, mutation V408M shown to be responsible for a relatively severe FH phenotype in approximately 20% of affected Afrikaners (Kotze et al. 1991, 1993b), causes the receptor to be rapidly degraded leading to less than 2% of normal receptor activity (Fourie et al. 1988).

In this study the contribution of these mutations to the phenotypic expression of homozygous FH was studied in the genetically homogeneous Afrikaner population. We aimed to determine whether routine DNA diagnosis in the Afrikaner population should in future be extended to include other LDLR gene mutations or other loci recently implicated in primary hypercholesterolaemia (Haddad et al. 1999, Varret et al. 1999, Zuliani et al. 1999, Ciccarese et al. 2000), and secondly to assess whether mutation status may provide useful parameters of disease progression in homozygous FH.

MATERIALS AND METHODS

Patients

DNA samples of 4 FH homozygotes of Afrikaner lineage attending lipid disorder clinics in South Africa were subjected to extensive mutation screening, after exclusion of the three founder-related LDLR mutations, D154N (FH-3), D206E (FH-1) and V408M (FH-2). The diagnosis of homozygous FH in these patients was based on standard criteria (Raal et al. 1997a), including elevated plasma cholesterol concentration, the presence of tendon xanthomata, and a family history of premature CHD. Pretreatment levels of total plasma cholesterol (TC), high density lipoprotein cholesterol (HDLC) and triglycerides (TG) were determined as previously described (Kotze et al. 1987). Low density lipoprotein cholesterol (LDLC) concentration was calculated according to the

Friedewald equation [TC-(HDLC+TG/2.18]. Lipid profiles were compared in 47 molecularly characterised FH homozygotes from 36 kindreds grouped according to the presence or absence of the LDLR gene mutation, V408M. Only FH homozygotes of whom pretreatment lipid levels were available were included in this analysis. Clinical, biochemical and molecular characteristics have previously been described in the majority of these patients (Kotze et al. 1989a, 1991, Raal et al. 1997a).

DNA analysis

Genomic DNA amplified by the polymerase chain reaction (PCR) was subjected to combined heteroduplex-single strand conformation polymorphism (HEX-SSCP) analysis, following exclusion of the Afrikaner founder mutations, D154N, D206E and V408M, according to Kotze et al. (1995b). Patient samples were also screened for the common apolipoprotein (apo) B3500 mutation causing familial defective apolipoprotein B-100 (FDB) (Soria et al. 1989), by including an allele-specific primer set (Wenham et al. 1991) in the multiplex PCR assay. Direct DNA sequencing on an automated ABI 310 system was subsequently performed to identify mutations in PCR amplified products demonstrating electrophoresis mobility shifts.

Statistical analysis

Mixed-effects analysis of variance was used to compare the mutation groups with respect to differences in their mean lipid profiles. Adjustment for age, gender and familial effects were made and adjusted means calculated.

RESULTS

Mutation analysis

The mutation status of 74 Afrikaner FH homozygotes are summarised in table 5.1, together with that of 11 FH homozygotes from other population groups in South Africa. Although each population group has a unique spectrum of mutations, the most common LDLR gene mutation in South Africa (D206E) was also detected in a compound FH heterozygote of mixed Jewish/Afrikaner descent (Raal et al. 1997a). Only two of four relatively common missense mutations D200G, S258L, C356Y and G361V, previously identified in 14 apparently unrelated FH heterozygotes (Vergotine et al. 2001a), were detected in FH homozygotes. These mutations in exons 4 (D200G) and 6 (S258L) of the LDLR gene result in receptor-defective alleles (Hobbs et al. 1992). In one of the

Table 5.1 Spectrum of mutations in South African FH homozygotes

LDLR genotype	Reference	Number
Afrikaner population		
W66G/A370W*	#	1
D154N/D154N	#	1
D154N/D206E	Kotze et al. 1991, Callis et al. 2001a	3
D154N/V408M	Kotze et al. 1991	1
D200G/D206E	#	2
D206E/D206E	Kotze et al. 1991, Callis et al. 2001a	29
D206E/V408M	Kotze et al. 1991, Callis et al. 2001a	30
S285L/S285L	#	1
V408M/V408M	Kotze et al. 1991	6
Black population		
137del6/137del6	Leitersdorf et al. 1988, Thiart et al. 2000	2
Indian population		
M-21L/C371X	Langenhoven et al. 1996	1
D69Y/E119K	Rubinsztein et al.1993a	1
P664L/P664L	Rubinsztein et al.1992	2
S765C/S765C	Kotze et al. 1997	1
Jewish population		
D206E/N407K	Raal et al. 1997a	1
Mixed ancestry (coloured population)		
Del 2.5-kb/Del 2.5-kb	Henderson et al. 1988	2
European ancestry		
N543H/2394del9	#	1

Mutation names are given according to Beaudet et al. (1996) and Antonarakis et al. (1998). The majority of mutations summerised in this table are included in FH databases http://www.umd.necker.fr. and http://www.ucl.ac.uk/fh. * Stu I polymorphism

Previously-described mutations identified in South African FH homozygotes

four Afrikaner FH homozygotes subjected to extensive HEX-SSCP mutation analysis no LDLR gene mutation could be identified.

Genotype-phenotype correlation studies in FH homozygotes

Homozygous Afrikaner FH patients with mutations D154N, D206E (receptor-defective) and/or V408M (receptor-negative), of whom clinical and biochemical data were available, were subdivided into two groups according to the presence (n=24) or absence (n=23) of mutation V408M. In the receptor-defective group, 21 patients were homoallelic for mutation D206E and two heteroallelic for mutations D154N and D206E. In the V408M-positive group, five patients were homoallelic for this mutation, one a compound heterozygote with mutation D154N and 18 compound heterozygotes with mutation D206E. Mean ages were similar in the two groups and considerable variation in lipid profiles was observed within the groups. The youngest patient (1 year) was a compound heterozygote for mutations D206E and V408M, while the eldest patient (54 years) was homozygous for mutation D206E. The highest LDLC level (24.5mmol/l) was observed in a homoallelic FH homozygote with mutation V408M, while the lowest LDLC (9.9 mmol/l) was determined in a patient with two copies of the receptor-defective

Table 5.2 Comparison of lipid and lipoprotein concentrations in Afrikaner FH homozygotes with and without mutation V408M

	FH 2 mutation absent (7 males / 16 females)			FH 2 mutation present (6 males / 18 females)			
	Mean	Range	SD	Mean	Range	SD	P value
Age (years)	18.5	6-54	12.3	18.2	1–48	11.0	0.9823
TC (mmol/l)	18.0	10.6-25.5	3.2	20.6	12.7-25.7	3.3	0.0077
LDLC (mmol/l)	16.8	9.9-24.0	3.0	19.2	11.5-24.5	3.3	0.0150
HDLC (mmol/l)	0.6	0.1-1.0	0.2	0.8	0.4-2.0	0.3	0.1767
TG (mmol/l)	1.1	0.5-1.7	0.4	1.3	0.4-1.7	0.9	0.3351

FH, familial hypercholesterolaemia; TC, total cholesterol; LDLC, low density lipoprotein cholesterol; HDLC, high density lipoprotein cholesterol; TG, triglycerides.

mutation D206E. After adjusting for age and gender, as well as familial dependence, statistically significant differences were found between the mean TC ($P<0.008$) and LDLC ($P<0.015$) levels, while mean HDLC and TG levels did not differ significantly between the two groups (Table 5.2). Tendon xanthoma was present in all the FH homozygotes studied. The incidence of CHD tended to be higher in the patient group with mutation V408M (54% vs 48%), but this difference did not reach statistical significance.

DISCUSSION

Major advantages can be derived from knowledge of the existence of a founder effect underlying FH in a specific population. Most importantly, it allows presymptomatic diagnosis and preventive treatment of this potentially lethal disease. Enrichment for certain mutations presents an important resource, enabling one to (i) analyse the geographical distribution and origins of mutations and (ii) perform genotype-phenotype correlation studies and evaluate factors modulating phenotypic expression of the disease.

The mutation screening approach applied in this study, following the exclusion of three founder-related LDLR gene mutations underlying FH in approximately 90% of affected Afrikaners (Kotze et al. 1991), resulted in the identification of three LDLR gene mutations in four Afrikaner FH homozygotes. One patient was homozygous for mutation S285L and the other two heterozygous for mutations W66G and D200G, respectively. Failure to identify LDLR gene mutations in one of the FH homozygotes presenting with a TC level of 17.05 mmol/l, and in two other homozygous patients heterozygous for either mutation, D154N or D206E, may be related to limitations imposed by the mutation detection method used or the considerable locus heterogeneity underlying primary hypercholesterolaemia (Varret et al. 1999, Zuliani et al. 1999, Ciccarese et al. 2000). Further studies focusing on the possible involvement of gene loci other than the LDLR and APOB genes are warranted in the molecularly-uncharacterised South African patients, since this may lead to the identification of new molecular targets for intervention strategies to reduce the risk of premature atherosclerosis.

Another possibility that must be considered, is the likelihood that missense mutations representing polymorphisms or rare variants may contribute to the disease

phenotype, particularly in patients with clinical features suggestive of homozygous FH in the presence of a single gene defect. This may be the case in the Afrikaner FH homozygote with mutation W66G, since the StuI polymorphism (A370W) (Kotze et al. 1986) shown to have a mild effect on plasma cholesterol levels in the general population (Gudnason et al. 1995), was the only other sequence change detected in this patient despite extensive HEX-SSCP mutation screening. It seems likely that the rare variants such as T705I (Jensen et al. 1996, Lombardi et al. 1997) and N407K (Raal et al. 1997a, Callis et al. 2001b) may have similar subtle phenotypic effects that may become clinically relevant only in the presence of other disease-causing mutation(s).

Only two of the four missense mutations, D200G, S258L, C356Y and G361V, previously identified in more than one unrelated Afrikaner FH heterozygote (14 families) (Vergotine et al. 2001a), were detected in FH homozygotes. Mutation S258L was detected in a homoallelic FH homozygote and mutation D200G in a compound heterozygote (and a similarly affected family member) together with the most common Afrikaner founder-related mutation, D206E. In view of the fact that these mutations have previously been described in FH patients from the Netherlands (S258L) and the United Kingdom (D200G) (Hobbs et al. 1992, Gudnason et al. 1993), representing European populations that contributed to the gene pool of the Afrikaner (Botha and Beighton 1983), we postulate the occurrence of minor founder events. However, since both parents of the homoallelic FH homozygote with mutation S285L tested positive for this mutation, consanguinity cannot be ruled out in this Afrikaner family. The two exon 8 mutations, C356Y and C361V, identified in 6 apparently unrelated FH heterozygotes, most likely originated in South Africa. Notably, four distinct haplotypes associated with mutations, D200G, S258L, C356Y and G361V, have been defined by Kotze et al. (1989a) using ten LDLR gene polymorphisms prior to the application of sequence analysis to identify the gene mutations associated with the specific chromosomal backgrounds. Extended haplotype studies using two highly informative microsatellite markers flanking the LDLR gene (Thiart 2000) were consistent with common origins for mutations D200G, S258L and G361V, while mutation G356Y was associated with different alleles. The minor differences in allelic size associated with this mutation can probably be attributed to replication slippage within the repeats, since a recurrent mutational event within the same population is highly unlikely.

Defesche and colleagues (1993) provided evidence that the Afrikaner founder mutation V408M originated in the Netherlands, while mutation D206E (Gudnason et al. 1993), and possibly also D154N, were introduced into South Africa from the United

Kingdom. In a recent study by Callis and colleagues (2001a), it was shown that the distribution of these mutations in South Africa correlates with both home language (English or Afrikaans) and historical affiliation to a minor religious group, the Gereformeerde Church. This finding is consistent with a previous report by Kotze and co-workers (1991), suggesting that the distribution of the Afrikaner founder mutations may differ between geographical regions.

Previous studies have demonstrated significant differences in plasma cholesterol concentrations between Afrikaner adults heterozygous for receptor-negative (V408M) versus receptor-defective (D154N, D206E) LDLR mutations (Kotze et al. 1993a, Graadt van Roggen et al. 1995). However, in a study of 116 children with one of these founder-related LDLR gene mutations, no significant differences in lipid levels could be observed between children with receptor-defective versus receptor-negative mutation types. This finding is consistent with the notion that genetic and environmental influences, apart from the LDLR gene defect, are more important in determining lipid levels in FH heterozygous children than in adults (Kotze et al. 1993b).

The present study suggests that LDLR mutation status may provide useful parameters of disease expression in Afrikaners with homozygous FH. Comparison of lipid profiles demonstrated significantly higher TC and LDLC levels in FH homozygotes with the receptor-negative mutation V408M, compared with the receptor-defective patient group. In contrast to the study of Moorjani and colleagues (1993), where no overlap in plasma cholesterol levels was observed between French-Canadian homozygotes with mutation W66G (receptor-defective, ~25-100% activity) and a greater than 10-kb deletion (receptor-negative), considerable overlap was observed in Afrikaners with different mutations. This may be ascribed to a smaller difference in severity between the receptor-defective (D154N and D206E, ~ 20% receptor activity) and receptor-negative (V408M, <2% activity) (Fourie et al. 1988, 1992) phenotype compared with the two mutations analysed in the French-Canadian homozygotes (Leitersdorf et al. 1990, Hobbs et al. 1992). Another possibility could be the criteria on which the groupings were done, since the V408M-positive group included compound heterozygotes with both receptor-negative and -defective (D154N, D206E) mutations (due to the small number of V408M homozygotes available for this study). In a Japanese cohort of homozygotes, grouped according to LDLR activity of less than 2% versus 20-30% of normal, no significant differences in serum cholesterol concentrations between receptor-negative and receptor-defective types were observed (Yamamoto et al. 1989).

Mean plasma-cholesterol concentrations in FH homozygotes from various parts of the world are remarkably similar (Thompson et al. 1989), indicating that the differences in lifestyles known to affect plasma-cholesterol in the general population have no major effect in homozygotes (Moorjani et al. 1993, Slimane et al. 1993). As in the French-Canadian homozygotes (Moorjani et al. 1993), gender did not affect plasma cholesterol concentrations or the expression of CHD in the Afrikaner homozygotes. In the group of FH homozygotes studied by Yamamoto and colleagues (1989), the incidence of CHD was much higher among patients with the receptor-negative LDLR type than in those with the receptor-defective type (based on receptor activity), despite a lack of difference in plasma cholesterol concentrations. CHD and coronary deaths are more frequent in homozygotes in whom receptor activity is less than 2% of normal when compared with subjects in whom receptor activity is 20 to 30% of normal (Goldstein and Brown 1982), although such association was not observed in the Afrikaner patients. When patients homoallelic for mutation V408M were compared with FH homozygotes without this mutation, 40% of the former group presented with CHD, while 48% of the receptor-defective group had CHD. This may be due to treatment of homozygous FH, which complicates clinical comparison between groups. Association of mutation V408M with the highest risk (Kotze et al. 1993a) for developing premature CHD and the finding that the lipid-elevating effect of this receptor-negative mutation seems to be the most modifiable of the three Afrikaner founder mutations studied (Jeenah et al. 1993), nevertheless necessitates a more aggressive approach towards modification of risk factors in patients with mutation V408M.

The findings highlight the potential prognostic value of accurate DNA diagnosis in populations where a small number of mutations cause the disease in the vast majority of affected cases. Of the 85 South African FH homozygotes characterised by us and others (Table 5.1) at the molecular level, 74 were of Afrikaner lineage. The three founder mutations D154N, D206E and V408M were present in the majority (96% of defective alleles) of the Afrikaner FH homozygotes, while the most common mutation D206E was also detected in an FH homozygote of Afrikaner/Jewish descent. Inclusion of LDLR gene mutations D200G and S258L, together with the three common founder mutations D154N, D206E and V408M in routine DNA screening of Afrikaner FH patients, would allow mutation detection in more than 96% of affected cases. Since expression of CHD and coronary deaths are related to LDLR gene status (Goldstein and Brown 1982, Yamamoto et al. 1989, Moorjani et al. 1993), complete genotyping and accurate interpretation of genetic data are required for a reliable prognosis in

homozygous FH that is determined largely by mutational heterogeneity in the LDLR gene.

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CHAPTER 6

Evaluation of the angiotensin-converting enzyme gene as a modifier locus for familial hypercholesterolaemia in the genetically homogeneous Afrikaner population

ABSTRACT

Objective. Previous studies have demonstrated that the deletion/deletion (DD) genotype of the angiotensin-converting enzyme (ACE) gene may increase the risk of myocardial infarction and coronary heart disease (CHD) in patients with familial hypercholesterolaemia (FH). We determined the frequency of this polymorphism in South African Afrikaners with FH and population-matched controls, in an attempt to determine the appropriateness of multilocus genotyping in CHD risk assessment.

Methods. The genotype distribution of the ACE polymorphism was compared in 205 FH heterozygotes, 45 FH homozygotes and 215 controls (including 95 elderly subjects) of the same population. All the FH patients were heterozygous or homozygous for one of the founder-related low-density lipoprotein receptor (LDLR) gene mutations, D154N, D206E or V408M.

Results. The ACE DD genotype was detected at a significantly lower frequency in the FH heterozygotes ($P<0.004$) compared with younger controls. No significant differences were observed in FH homozygotes ($P<0.175$) (when compared with the younger control group) or between FH patients with and without CHD. Allelic distribution differed significantly between the two control groups ($P<0.031$), with the I-allele predominantly in the elderly.

Conclusions. Comparative analysis of molecularly-characterised FH patients and population-matched controls may represent a useful study approach to reveal clinically relevant allelic differences. The potential for different degrees of linkage disequilibrium of the ACE insertion/deletion (I/D) polymorphism with other potentially significant sequence changes in the gene and the modest degree of increased risk for CHD

associated with the ACE DD genotype, argues against the likelihood of this marker being a clinically useful indicator of increased risk for CHD in Afrikaner FH patients.

KEYWORDS: familial hypercholesterolaemia, angiotensin-converting enzyme polymorphism, Afrikaner population

INTRODUCTION

Cardiovascular disease (CVD) is a complex multifactorial condition caused by an interaction of various genetic and environmental factors. Familial hypercholesterolaemia (FH) has been recognised as a common cause of premature coronary heart disease (CHD), particularly in South Africa where FH is extremely common due to population enrichment of multiple founder-type low density lipoprotein receptor (LDLR) gene mutations (Kotze et al. 1991, 1997, Meiner et al. 1991, Loubser et al. 1999, Thiart et al. 2000). In general, 50% of men and 20% of women with heterozygous FH have clinical evidence of CHD by age 45 (Mabuchi et al. 1989, Kane et al. 1990, Seed et al. 1990, Goldstein et al. 1995). Males diagnosed with FH have a 40-fold increased risk of premature CHD, and if left untreated, their life expectancy is reduced by 20 to 30 years (Williams et al. 1993a). Patients with the severe homozygous form of FH usually die of a heart attack before the age of 20 years.

The mutational heterogeneity of the LDLR gene appears to explain most of the clinical variation found among FH homozygotes (Sprecher et al. 1985, Moorjani et al. 1993). Although LDLR type also correlates with phenotypic expression of heterozygous FH (Kotze et al. 1993a, Gudnason et al. 1994), interaction with various environmental and genetic factors may greatly influence LDL cholesterol status in association with a specific mutation and clinical outcome (Kotze et al. 1993b, 1998b, Pimstone et al. 1998). Various candidate factors have been investigated as potential modifiers of the FH phenotype, including the genes responsible for variation in apolipoprotein E (apo E), lipoprotein(a) [Lp(a)], methylenetetrahydrofolate reductase (MTHFR) and the angiotensin-converting enzyme (ACE) levels.

Most of the investigations on the effect of other genes on CHD risk in FH patients provided inconclusive or contradictory results which, according to Scholtz and colleagues (2000a) who studied the role of Lp(a) in the genetically homogeneous

Afrikaner population of South Africa, may be related to various confounding factors. Small sample size and the composition of the FH patient cohort that may consist of young individuals who may still develop CHD, and/or survivors without additional risk factor(s) (that may have led to early coronary deaths in a subgroup of patients) are important considerations. The difficulty to demonstrate the effect of a minor gene in the presence of a major gene may also explain the failure of indicating an association between known risk factors and CHD in unrelated FH patients, since many different risk factors may be involved in the respective families being studied. This notion was substantiated by earlier findings of Kotze and co-workers (1993a) studying the apo E polymorphism in molecularly-characterised Afrikaner FH patients, where the expected allelic effects on plasma cholesterol levels, as confirmed in the Afrikaner control group, could not be observed in an additive manner in the FH patients. While comparative studies of CHD risk factors in FH patients with varying degrees of disease expression can therefore be misleading, negative results should not detract from the potential importance of multilocus risk assessment in FH.

Identification of modifiable genetic risk factors such as mutations in the MTHFR gene causing mild hyperhomocysteinaemia (Frosst et al. 1995) may be of considerable importance in the management of FH patients (Tonstad et al. 1997). This, not withstanding the fact that the potential effect of functional polymorphisms in the gene may not be evident in FH patients (Defesche et al. 1998) where LDLR gene mutations represent the major determinants of CHD risk. Analysis of the MTHFR gene in the Afrikaner population has also not revealed significant differences in allelic distribution of mutations, 677C→T and 1298A→C, between FH patients with and without CHD, although significantly lower frequencies of these variants were observed in a population-matched control group (Scholtz 1998, Scholtz et al. 2001). These findings raised the possibility that comparison of allelic frequencies between mutation-positive patients and appropriate control groups may provide an alternative means of identifying modifier loci in FH. This approach has recently been applied in the analysis of another common autosomal dominant disease in the Afrikaner population, namely variegate porphyria (VP) which displays low disease penetrance, most likely as a consequence of gene-gene (de Villiers et al. 1999) and/or gene-environment interaction in relevant families. Subsequent segregation analysis in two relatively large VP families with the relevant gene mutations (Kotze et al. unpublished data) was consistent with the notion that the

key to unravelling the various factors involved in the phenotypic expression of FH (Kotze et al. 1993a) is to be found in families where the clinical manifestation of the disease varies among relatives.

This investigation focused on evaluation of the angiotensin-converting enzyme (ACE) gene as a modifier locus for FH, since two separate studies have demonstrated that patients with the deletion-allele of the ACE ID polymorphism have an increased risk of developing CHD (Petrovic et al. 1996, O'Malley et al. 1998). We therefore determined the frequency of the ID polymorphism in the homogeneous Afrikaner population of South Africa, in an attempt to establish the usefulness of comparative analysis within and between patient and control groups. The identification of modifier genes that may influence the clinical expression of common single gene disorders is of fundamental importance for genetic counselling and treatment strategies.

MATERIALS AND METHODS

Patients

Blood samples were obtained, with informed consent, from 250 apparently unrelated Afrikaner index patients previously diagnosed with FH at both the clinical and molecular level (Kotze et al. 1991, 1995b). Two hundred and five patients were heterozygous for one of the three Afrikaner founder-related LDLR gene mutations, D154N (19), D206E (145) or V408M (41) (Kotze et al. 1991). The 45 FH homozygotes included in the study had two of the founder-related LDLR gene defects (Kotze et al. 1995a) and were categorised into CHD-positive and CHD-negative FH-groups (Table 6.1). Seventy-five patients were recorded as having CHD. They presented with angina pectoris, suffered a myocardial infarction or underwent coronary artery bypass grafting / angioplasty. All the FH heterozygotes, 75 males and 130 females, were above the age of 25 years. DNA of 120 healthy blood donors, students and unaffected spouses of index patients as well as 95 elderly subjects (>70 years), all from Afrikaner lineage, were used as control samples. Levels of total plasma cholesterol (TC), high density lipoprotein cholesterol (HDLC) and triglycerides (TG) were determined as previously described (Kotze et al. 1987). Low density lipoprotein cholesterol (LDLC) concentration was calculated according to the Friedewald equation LDLC = [TC-(HDLC+TG/2.18].

Table 6.1 Characteristics of FH heterozygotes with and without CHD

	CHD-positive	CHD-negative	P value
Number of patients	75	130	
Male / Female ratio	38 / 37	37 / 93	0.002
Mean age (years)	52.31 (11.30)	46.20 (13.01)	0.001
Mean TC (mmol/l)	9.24 (2.21)	9.24 (1.90)	0.606
Mean LDLC (mmol/l)	7.45 (2.11)	7.48 (1.87)	0.741
Mean HDLC (mmol/l)	1.07 (0.38)	1.12 (0.36)	0.782
Mean TG (mmol/l)	1.82 (1.35)	1.44 (0.82)	0.071
D154N mutation (n)	5 (6.7%)	14 (10.8%)	
D206E mutation (n)	50 (66.7%)	95 (73.1%)	
V408M mutation (n)	20 (26.7%)	21 (16.15%)	0.152*

* Refers to mutation distribution

Standard deviations and representative percentages, where appropriate, are noted in parentheses. FH, familial hypercholesterolaemia; D154N, D206E, V408M, Afrikaner founder-related LDLR gene defects; TC, total cholesterol; LDLC, low density lipoprotein cholesterol; HDLC, high density lipoprotein cholesterol; TG, triglyceride; n, number.

Mutation detection

Genomic DNA was extracted from whole blood (Miller et al. 1988) and the ACE genotypes were determined by allele-specific polymerase chain reaction (PCR) (Rigat et al. 1992, Tiret et al. 1992). Due to various reports on possible mistyping of the DD allele (Shanmugan et al. 1993, Fogarty et al. 1994, Odawara et al. 1996, 1997), two additional primer sets were used (Lindpainter et al. 1995), and 5% (vol/vol) dimethyl sulfoxide (DMSO) was included in the final reaction mixture.

Statistical analysis

Chi square (χ^2) values were calculated and their significance levels determined by two-way contingency tables. P values <0.05 were regarded as statistically significant. Mixed-effects analysis of variance was used to compare the mutation groups with respect to differences in their mean lipid profiles. Adjustment for age, gender and familial effects were made and adjusted means calculated.

RESULTS

No statistically significant differences were observed in the mean TC, LDLC and HDLC levels between the CHD-positive and CHD-negative groups, while the mean TG levels were marginally but significantly higher (P<0.07) in the CHD-positive group (Table 6.1). The mean age of the CHD-positive group was higher than in the CHD-negative group. The male/female ratio was approximately 1:1 in the CHD-positive group, while a ratio of 1:2.5 was observed in the CHD-negative group. Mutation V408M, associated with the most severe FH phenotype (<2% of normal receptor activity), predominated in the CHD-positive group. Many other possible confounding factors have not been determined in the two study groups.

No correlation was observed between the ACE DD polymorphism and CHD within the three Afrikaner founder-related LDLR gene mutation groups (data not shown). The risk of CHD in association with the ACE DD genotype was not increased in FH homozygotes and FH heterozygotes (P<0.746, χ^2 =0.11) (Table 6.2). A statistically significant difference was observed when comparing the DD, ID and II genotypes between the FH heterozygous patients and the healthy controls (P<0.014, χ^2 = 8.48). These data became even more significant (P<0.004, χ^2 = 8.47) when comparing the ratio of the DD genotype to the ID+II genotypes. A significant difference was observed between the CHD-positive group and the control group subjects (P<0.029, χ^2 = 4.78) when comparing the frequencies of the DD genotype to the ID+II genotypes. No statistically significant difference was observed between the FH homozygotes and the control group (P<0.175, χ^2 =1.84). Although the difference between the FH groups and

Table 6.2 Comparison of genotype distribution and allele frequencies of the ACE I/D polymorphism between FH groups and control individuals

Genotype	FH homozygotes	FH heterozygotes			Control samples	
	Unrelated	All	CHD-negative	CHD-positive	Elderly	Controls
	n=34	n=205	n=130	n=75	n=95	n=120
II	7 (21%)	29 (14%)	20 (15%)	9 (12%)	19 (20%)	14 (11.7%)
ID	18 (53%)	127 (62%)	79 (61%)	48 (64%)	52 (54.7%)	59 (49.2%)
DD	9 (26%)	49 (24%)	31 (24%)	18 (24%)	24 (25.3%)	47 (39.2%)
P value - Controls vs:	0.175	0.004	0.009	0.029	0.031	
P value – Elderly vs:	0.891	0.799	0.806	0.849		0.031
Allele						
I – allele	0.47	0.45	0.46	0.44	0.47	0.36
D – allele	0.53	0.55	0.54	0.56	0.53	0.64
P value – Controls vs:	0.106	0.027	0.031	0.127	0.020	
P value – Elderly vs:	0.964	0.607	0.736	0.536		0.020

ACE, angiotensin-converting enzyme; FH, familial hypercholesterolaemia; CHD, coronary heart disease;

D, deletion allele; I, insertion allele; P values calculated for DD > II + ID

the elderly control group was statistically insignificant, this was significant when the elderly and younger control groups were compared ($P<0.031$, $\chi^2 = 4.63$). The ACE DD genotype was not associated with the presence of CHD in males or females separately, or within heterozygous patients aged, 40 to 60 years (Table 6.3).

Table 6.3 ACE genotype and incidence of CHD in patients with heterozygous and homozygous FH

Male		Female	
Genotype	**n (%) CHD**	**Genotype**	**n (%) CHD**
All FH heterozygotes (n=205)			
II (n=13)	4 (31%)	II (n=16)	5 (31%)
ID (n=45)	26 (58%)	ID (n=82)	22 (27%)
DD (n=17)	8 (47%)	DD (n=32)	10 (31%)
II + ID (n=58)	30 (52%)	II + ID (n=98)	27 (27.5%)
FH heterozygotes aged 40-60 years (n=104)			
II (n=4)	2 (50%)	II (n=4)	2 (50%)
ID (n=26)	19 (73%)	ID (n=40)	11 (27.5%)
DD (n=9)	6 (67%)	DD (n=21)	7 (33%)
II + ID (n=30)	21 (70%)	II + ID (n=44)	13 (30.0%)
FH homozygotes (n=44)			
II (n=1)	0 (0%)	II (n=6)	1 (17%)
ID (n=9)	3 (33%)	ID (n=16)	6 (38%)
DD (n=3)	1 (33%)	DD (n=9)	6 (67%)
II + ID (n=10)	3 (30%)	II + ID (n=22)	7 (32%)

CHD, coronary heart disease; FH, familial hypercholesterolaemia

DISCUSSION

The frequencies of the ACE I/D gene polymorphism were determined in a cohort of Afrikaner FH patients in order to evaluate their potential in predicting CHD risk in a homogeneous already high-risk population. Since the molecular basis of FH is largely elucidated, this common autosomal co-dominant disorder can serve as a model for studying interaction with candidate modifier genes such as the ACE gene. The relatively homogeneous Afrikaner population with its high prevalence of FH (1/70) may be particularly suitable for analysis of gene-gene and gene-environment interaction, since a limited number of LDLR gene defects (D154N, D206E and V408M) are responsible for the disease in more than 90% of all clinical FH cases (Kotze et al. 1991). We argued that a comparison of Afrikaner FH patients with unselected healthy Afrikaner subjects from the same population would reveal clinically relevant allelic differences only if the ACE I/D polymorphism is a major contributor to CHD risk in the general population. Although a statistically significant difference was observed between unselected healthy Afrikaners and FH heterozygotes of Afrikaner lineage ($P<0.004$) no association was observed in FH homozygotes ($P<0.175$). Failure to demonstrate significant differences between the CHD-positive and CHD-negative groups most likely reflect the presence of many confounding factors. There were more males in the CHD-positive group, their mean age was higher and they had significantly higher TG levels compared with the CHD-negative group.

Although a higher frequency of CHD was observed in FH heterozygous males (in both age groups), no association between the ACE DD polymorphism and the presence of CHD was observed in both male and female patients (data not shown) (Table 6.3). In contrast, this was reversed in FH homozygotes where the female patients presented with a higher frequency of CHD related to the D allele, although no statistical difference was observed. This may be the result of a small sample size.

The ACE gene located on chromosome 17q23 (Mattei et al. 1989) has been identified as a risk factor for the development of CVD (Nakai et al 1994, Ruiz et al. 1994, Gardeman et al. 1995, 1998, Wang et al. 1996) and myocardial infarction (MI) (Cambien et al. 1992, 1994, Tiret et al. 1993, Ludwig et al. 1995), while others state the contrary (Lindpainter et al. 1995, Ludwig et al. 1995, Agerholm-Larsen et al. 1997, Ferrières et al. 1999). This polymorphism, a 287-base pair (bp) Alu-repeat in intron 16, is co-dominantly associated with the level of circulating ACE enzyme, with almost half of the phenotypic variation being attributable to the segregation of the ACE I/D allele

(Rigat et al. 1990). The ACE DD genotype is associated with almost double the values of plasma concentrations of circulating ACE (Rigat et al. 1990), which result in the enhanced conversion of angiotensin I into angiotensin II, which inactivates bradykinin (Cambien et al. 1992). Angiotensin II increased the influx of ^{125}I-labelled LDL into the arterial wall of rabbits (Nielsen et al. 1994), with the net result being the proliferation of smooth muscle cells and vasospasm (Cambien et al. 1992).

Controversy exists about the link between the deletion variant of the ACE gene and increased risk for CHD and myocardial infarction (MI) (Singer et al. 1996, Butler et al. 1997). Various reviews, summarising all the published studies until 1997, regarding the association of the ACE I/D polymorphism and the risk of CHD and MI, have been published (Butler et al. 1997, Schunkert 1997, Staessen et al. 1997). Butler and colleagues (1997) argued that the DD genotype adversely influences CHD, but appears to do so in specific geographical areas and in particular patient subgroups. They reported a positive association between the DD genotype and MI in nine case studies, a positive correlation between the DD genotype and CHD in three case studies, no correlation between the genotype and MI in three cases and a negative correlation with MI in one case report. They concluded that the impact of the DD genotype appears to be small and its clinical manifestations rather heterogeneous. Staessen and colleagues (1997) reported a positive association between the ACE DD genotype and MI in various countries, from homogeneous subgroups as well as the general population.

Schunkert (1997) evaluated the criteria for CHD and observed a positive correlation in homogeneous subgroups (e.g. non-insulin-dependent diabetes mellitus (NIDDM) patients and the Asian population), but state that the overall picture was inconclusive. In a more recent review, O'Malley and colleagues (1999) acknowledged the heterogeneity in the association between the ACE DD genotype and CHD, and suggested separation of study groups according to population. Although the ACE DD genotype appears to be associated with an increased risk for CHD in many populations, this association is heterogeneous in populations of different geographical origins. They further argue that even if the ACE I/D polymorphism itself was the cause of an increased propensity for myocardial infarction, its effects are expected to be moderated by differing genetic backgrounds, and the increased risk may be gender-dependent in some populations. This notion was confirmed by the findings of Galinsky and co-workers (1997) where significant differences were observed between the ACE genotype in elderly men and women. O'Malley and colleagues (1999) concluded that this might be expected from a polymorphism suspected of being a genetic marker in linkage

disequilibrium with an underlying disease-associated mutation. The degree of linkage disequilibrium is not expected to be uniform in all populations (O'Malley et al. 1999). The protective nature of the TT genotype of the ACE A240T promoter polymorphism towards myocardial infarction is most interesting, since this allele appears to be in positive linkage disequilirium with the D allele of the ACE I/D polymorphism (Foy et al. 1997, O'Malley et al. 1999).

The clinical consequences of an increased risk for CHD associated with the ACE DD genotype in an already high-risk population are quite severe. The incidence of CHD in FH heterozygous males with the ACE DD genotype was 2.2 times higher than those who had the ACE ID/II genotypes (O'Malley et al.1998). Petrovic and colleagues (1996) reported an excess of the ACE DD/ID genotypes in CHD-positive versus CHD-negative FH patients. Patients with FH as well as the ACE DD genotype may therefore have an increased risk for developing CHD. The association of the ACE DD genotype with an increased risk for MI and CHD in a high-risk subgroup of NIDDM patients was similar to that observed in certain general population studies (O'Malley et al. 1999).

Failure to demonstrate significant differences in allelic distribution between FH heterozygotes with or without CHD might reflect the difficulty of defining minor gene effects in the presence of a major gene effect. However, the potential for different degrees of linkage disequilibrium of the ACE I/D polymorphism with other potentially significant sequence changes in the gene and the modest degree of increased risk for CHD associated with the ACE DD genotype, argues against the likelihood that this marker is a clinically useful indicator of increased risk for CHD in Afrikaner FH patients. This view was supported by the finding that the I-allele, and not the DD genotype previously shown to be associated with longevity (Galinsky et al. 1997; Luft 1999), was increased in the elderly Afrikaner population compared with younger controls ($P<0.02$) or the FH patient groups. It therefore seems likely that in the genetic background of the Afrikaner, the ACE II genotype is associated with longevity. Analysis of the promoter region of the ACE gene may in future reveal whether mutation(s) affecting transcriptional activity may modify the risk for CHD in the South African population.

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CHAPTER 7

CONCLUSIONS

Molecular diagnosis of familial hypercholesterolaemia in the genetically distinct populations of South Africa

Inherited lipid and lipoprotein disorders contribute significantly to the high mortality rate from coronary heart disease (CHD) in Westernised countries. Familial hypercholesterolaemia (FH) is a major risk factor for CHD and the high prevalence of this disease in several genetically distinct South African populations has important health implications. Molecular research performed on these groups over a period of more than 15 years, focussed on delineation of the spectrum of disease-related mutations in South Africa and the development of cost-effective methods (Kotze et al. 1995b, Appendix 2) to identify relevant gene defects in the low density lipoprotein receptor (LDLR) and apolipoprotein B genes. A family-based approach is used for presymptomatic diagnosis of FH in families, following the identification of the causative mutation in the index patient. More recently, the emphasis has shifted to the identification of other loci that may influence the expression of FH in affected South Africans (Scholtz 1998, Scholtz et al. 2000a, 2001, this study), in order to identify those individuals at highest risk of developing premature CHD. Accurate disease diagnosis is a prerequisite for optimal treatment of FH, particularly when considered within the context of potential modifier loci and environmental risk factors that can be avoided.

The present study was aimed at collecting information to improve the molecular diagnostic service and clinical management of FH. Knowledge that specific mutations predominate in the South African Afrikaner (Chapter 3) and Jewish (Chapter 4) populations facilitated rapid molecular characterisation of FH index cases and their close relatives. Mutation analysis of patients from heterogeneous populations (Chapter 2), on the other hand, involved a more generalised approach of screening the entire LDLR gene. Mutation analysis in FH homozygotes demonstrated that mutation status may provide useful parameters on which to base predictions of clinical progression,

even in these severely affected patients (Chapter 5). Finally, the issue of potential modifier loci in FH was addressed (Chapter 6), which demonstrated that genetic risk factors may differ between populations. A polymorphic variant implicated in cardiovascular disease should therefore be evaluated within a specific population context before it can be applied as a genetic marker for disease risk.

7.1 MUTATIONAL SPECTRUM AND GEOGRAPHICAL DISTRIBUTION

The mutational spectrum underlying lipid abnormalities differs among population groups. Knowledge of the spectrum of gene defects causing primary hypercholesterolaemia in a specific population and in affected families allows accurate disease diagnosis and preventive treatment. An efficient diagnostic strategy depends on the genetic composition of the relevant population (Botha and Pritchard 1972).

While familial defective apolipoprotein B (FDB) appears to be extremely rare in the South African population (Rubinsztein et al. 1994, 1995, this study), up to 80 different LDLR gene mutations underlying FH have been identified locally (Thiart 2000, This study). These were detected in Afrikaner (Kotze et al. 1991, Vergotine et al. 2001a), Jewish (Meiner et al. 1991, this study), Indian (Rubinsztein et al. 1992, 1993a, Kotze et al. 1997), Coloured (Loubser et al. 1999) and Black (Thiart et al. 2000) populations, as well as recent immigrants living in South Africa (Callis et al. 1998, this study). The presence of multiple founder-type LDLR gene defects in the genetically distinct populations of South Africa (Table 7.1) enhances the prospects of DNA-based diagnosis of FH in this country. The wide spectrum of mutational events in patients from heterogeneous populations, such as the patient group studied by Callis and colleagues (1998, Chapter 2), indicated a more generalised approach of screening the entire LDLR gene for disease-related mutations, followed by family-based screening.

The exceedingly high death rate from premature CHD and coronary deaths in the Afrikaner population of South Africa (Wyndham 1978, Pretorius 1983) is undoubtedly a consequence of multiple founder-related LDLR gene mutations underlying the FH phenotype. Three LDLR gene mutations (D154N, D206E and V408M) account for approximately 90% of all clinical FH cases in the Afrikaner (Graadt van Roggen et al. 1991, Kotze et al. 1991, Steyn et al. 1997). Inclusion of mutations D200G, S285L, C356Y and G361V, that together account for heterozygous FH in approximately 6% of cases in routine DNA screening, would improve the diagnostic service for FH in Afrikaners. Implementation of a routine diagnostic service for FH at Universitas

Table 7.1 Spectrum of relatively common LDLR gene mutations in the genetically distinct populations of South Africa

LDLR gene mutation	Heterozygote frequency	Origin/Found in	References
AFRIKANER POPULATION			
D154N	10%	United Kingdom	Kotze et al. 1989b
D206E	60%	United Kingdom	Kotze et al. 1990
V408M	20%	Netherlands	Kotze et al. 1989b
D200G	2%	United Kingdom	Thiart 2000
S285L	1%	France	Thiart 2000
C356Y	2%	South Africa	Thiart 2000
G361V	1%	South Africa	Thiart 2000
COLOURED POPULATION			
D154N	0.5%	United Kingdom	Loubser et al. 1999
D206E	8%	United Kingdom	Loubser et al. 1999
V408M	5%	Netherlands	Kotze et al. 1995a
Del 2.5-kb	4%	South Africa	Henderson et al. 1988
652delGGT	3%	Lithuania	Loubser et al. 1999
P664L	2%	India	Loubser et al. 1999
BLACK POPULATION			
137del6	28%	South Africa	Leitersdorf et al. 1988 Thiart et al. 2000
INDIAN POPULATION			
P664L	50%	India	Rubinsztein et al. 1992 Kotze et al. 1997
JEWISH POPULATION			
652delGGT	80%	Lithuania	Meiner et al. 1991 This study

References do not necessarily refer to the first report of the specific mutation, but to detection in the South African population.

Hospital, using a population-based screening strategy (Kotze et al. 1995b, Appendix 2), resulted in a definite diagnosis in 44% (208/474) of all patient referrals. The relatively low mutation detection rate can be ascribed to the fact that most patients were referred on the basis of elevated plasma cholesterol levels, to confirm or largely exclude a diagnosis of FH. The distribution of the Afrikaner founder-related mutations in the five distinct geographical regions analysed in this study was in general agreement with that described previously for two geographical areas within South Africa (Graadt van Roggen et al. 1991, Kotze et al. 1991). Mutation D206E (FH-1) was most prevalent (63% combined average), followed by V408M (FH-2) (31.4%) and D154N (FH-3) (5.6%). The relative frequencies of the three Afrikaner founder-related mutant alleles correlated significantly with the distribution of both home language (English and Afrikaans) and historical affiliation with the Gereformeerde Church. Mutations D154N and D206E were most prevalent in the Western Cape and Gauteng regions where English is spoken by the majority of the residents. The Dutch-associated mutation V408M predominates in the Free State, Northern Cape and North-West provinces ($P<0.05$), where the largest percentage of the Afrikaans-speaking residents of South Africa are located. These findings would not affect the general mutation screening strategy in the Afrikaner population, since all three mutations were detected at relatively high frequencies in the different subgroups and can be detected simultaneously using a multiplex assay (Kotze et al. 1995b, Appendix 2). However, knowledge that a patient is affiliated with the Gereformeerde Church may be an incentive to first screen for the presence of mutation V408M.

The three Afrikaner founder-related mutations, D154N, D206E and V408M, contribute significantly (15%) to the FH phenotype in the indigenous South African Coloured population of mixed ancestry (Loubser et al. 1999). Although the prevalence of FH has not been determined in this group, the clinical impression is that it is relatively high. The three Afrikaner founder mutations, as well as the mutations found to be common in the Jewish (Meiner et al. 1991) and Indian (Rubinsztein et al. 1992) populations of South Africa, were probably introduced into the Coloured population by recent admixture. Predominance of mutations P664L and 652delGGT in the Indian (Kotze et al. 1997) and Jewish (this study) populations, respectively, was confirmed in follow-up studies including larger patient samples. A 6-bp deletion (137del6) in exon 2 of the LDLR gene, that predominates (28%) in Black FH patients in South Africa (Thiart et al. 2000), has not been detected in the Coloured FH patients studied. It has been

suggested that the virtual absence of FH in Africans may be due to altered clinical expression (Thiart et al. 2000) as a result of other genetic and environmental factors, including a prudent diet (Vermaak et al. 1991). These findings support the notion that clinical and biochemical criteria for the diagnosis of FH needs to be different by country/population (Pimstone et al. 1998), and stress the importance of DNA-based assays that allow accurate disease diagnosis in affected families. Similar to the distribution of the three Afrikaner founder mutations, the LDLR gene mutations shown to be relatively common in the different South African population groups (Table 7.1), have been detected in patients from various geographical regions in South Africa.

Failure to identify disease causing-mutations in all the clinically diagnosed FH patients was initially ascribed to the limitations imposed by the combination of mutation screening strategies applied. The identification of a third, and the proposal of even a fourth, major locus for autosomal dominant hypercholesterolaemia (ADH) (Varret et al. 1999), raise the possibility that these genes may underlie the disease phenotype in some of the molecularly uncharacterised FH patients. It is uncertain what percentage of FH in South Africa is related to these novel loci, which are expected to predispose to a relatively mild form of ADH in most cases and may therefore usually not be considered for extensive mutation screening.

Based on the data obtained in this and related studies, it is recommended that the common FH mutations identified in the respective genetically distinct populations of South Africa (Table 7.1), should be targeted as a first step in a population-directed screening strategy for FH, followed by tracing of the defective allele in family members. This approach is outlined in figure 7.1.

7.2 DNA DIAGNOSIS IN RELATION TO PHENOTYPIC VARIABILITY

In South Africa, the family-based approach performed as part of the genetic service to identify FH patients irrespective of clinical expression of the disease, has been extended to identify mutation-positive relatives of patients suffering from several genetic diseases that occur at increased frequencies due to founder gene mutations (Kotze and Callis 1999, Appendix 1). This case finding approach in FH involves the steps outlined in figure 7.2.

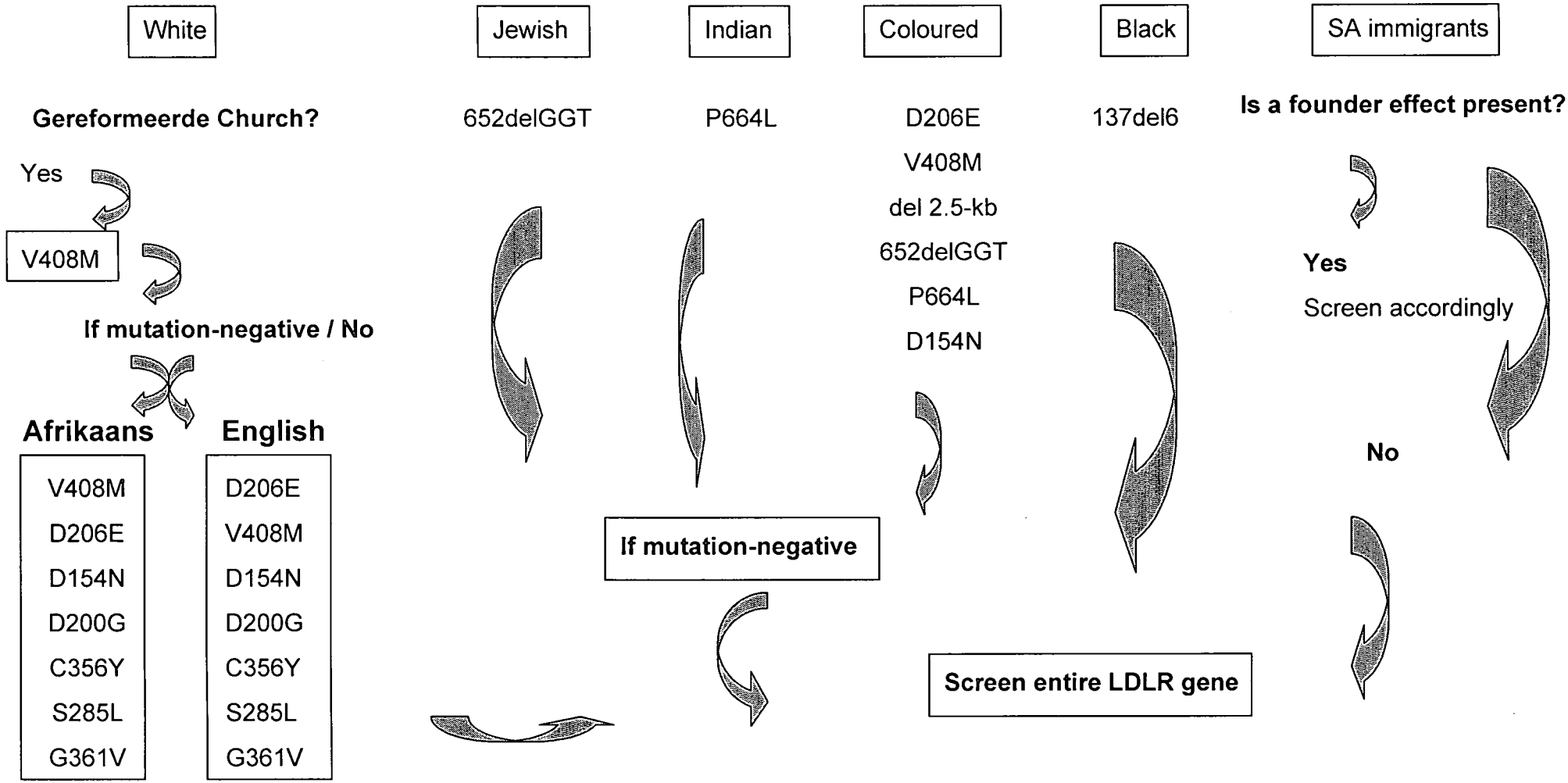

Fig. 7.1 Guidelines for population-directed screening in the genetically distinct populations of South Africa

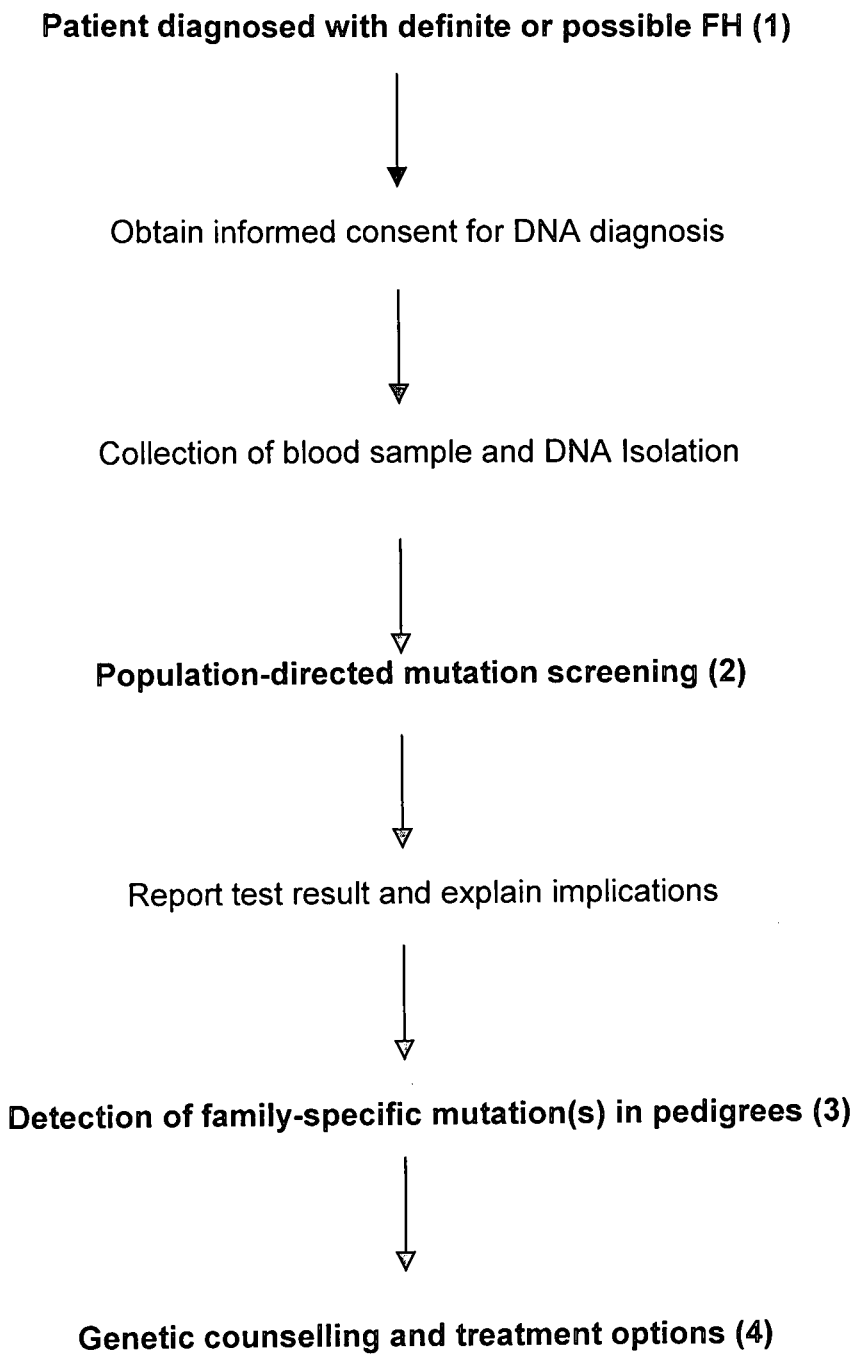

Fig. 7.2 Flow diagram for population-directed DNA diagnosis of FH in the South African population. (1) Referring doctor initiates identification of possible FH patient. The different issues that have to be discussed with the patient prior to collection of a blood sample for DNA analysis, is outlined in Chapter 1, section 5.4. (2) Mutation screening in a specific population group is performed according to the spectrum of LDLR gene mutations outlined in Table 7.1, by following the guidelines in figure 7.1. (3) Mutation detection in the index case leads to contact with relatives to offer/perform testing. (4) Efficient contact with the referring doctor / families allows appropriate genetic counselling and implementation of preventative treatment.

Step 1: *Index case finding.* This is the first person identified as having FH in the family. Index patients are referred for DNA screening from different sources such as lipid clinics, general practitioners, hospitals, mass population screening, and finally patient self-referral through media and publicity events.

Step 2: *Diagnosis.* Qualified persons review patient data and family history and make a diagnosis based on biochemical and/or mutation data.

Step 3: *Enter in FH registry.* The mutation data are entered into the database or FH registry and the referring doctor / FH patient is notified of the test results obtained.

Step 4: *Relative case finding.* Mutation-positive index cases are requested to ask family members to possibly undergo mutation screening or to give consent that relatives known to have high plasma cholesterol levels and/or early CHD may be contacted for this purpose. Family information is usually obtained from the index case.

Step 5: *Continuation of relative case finding.* After identifying family members with FH, several new FH cases can be found among more distant relatives. The probability of a family member carrying an LDLR mutation is well defined; the likelihood of having FH is 50% for each parent, sibling and child; 25% for each aunt, uncle, grandparent, niece and nephew; 12.5% for first cousins and siblings of grandparents.

In clinical practice the criteria for diagnosis of FH is based mainly on clinical findings, cholesterol concentrations and family history (Umans-Eckenhausen et al. 2001). The use of biochemically-defined cut-off points may, however, result in misdiagnosis of FH (Bhatnagar et al. 2000, Kastelein 2000, Umans-Eckenhausen et al. 2001). Active screening for a disorder requires accurate diagnosis, and in the case of FH this can only be provided by using DNA testing to identify the causative mutation (Kastelein 2000). The value of DNA diagnosis has been demonstrated repeatedly in the South African population (Kotze et al. 1993a, 1993b), particularly since it allows accurate presymptomatic (Kotze et al. 1998b, Vergotine et al. 2001a) and prenatal (Vergotine et al. 2001b) diagnosis of FH. An excellent example in this regard was provided in the study of the Afrikaner-Jewish family (Chapter 4). Both mutation-positive children of the compound heterozygous FH index patient with an apparently mild form of clinical homozygous FH, initially presented with plasma cholesterol values within the normal range, but three years later this was elevated above the 90th percentile for age and gender in one of the children willing to undergo testing. Plasma cholesterol levels may therefore not always be diagnostic of FH, although a combination of biochemical and molecular methods is most informative. A DNA-based screening programme for FH in

the Netherlands emphasised the need for an accurate and presymtomatic diagnosis of this treatable monogenic disorder (Umans-Eckenhausen et al. 2000).

Mutational heterogeneity of FH appears to explain most of the clinical variation found among FH homozygotes (Yamamoto et al. 1989, Moorjani et al. 1993) and may also account for some phenotypic variability in heterozygous FH (Kotze et al. 1993a, 1993b, Gudnason et al. 1994, Pimstone et al. 1998). Determination of lipid profiles in Afrikaner FH homozygotes with the receptor-negative mutation, V408M, in comparison with a receptor-defective patient group without this mutation, support the notion that LDLR mutation status may provide some indication of disease expression in Afrikaners with homozygous FH.

7.3 MODIFIER LOCI AND CLINICAL MANIFESTATION OF FH

The identification of modifier genes that may influence the clinical expression of single gene disorders is of fundamental importance for genetic counselling and treatment strategies. The wide spectrum of phenotypic variability detected in FH patients sharing the same LDLR defective allele (Kotze et al. 1993a, 1993b, Ferrières et al. 1995, Pimstone et al. 1998) suggests that other important predictors of CHD risk in FH remain to be identified. Thus, one challenge is to identify the common multilocus profiles that confer a high risk of cardiovascular disease within a specific population, and a second challenge is to understand how environmental factors modulate expression of a genetic predisposition to disease.

Analysis of the angiotensin converting enzyme (ACE) insertion-deletion (I/D) polymorphism is of particular importance in the high-risk FH population, where co-inheritance with FH-causing mutations may have severe clinical consequences (Petrovic et al. 1996, O'Malley et al. 1998, 1999). Failure to demonstrate significant differences in allelic distribution between Afrikaner FH heterozygotes with and without CHD might reflect the difficulty of defining minor gene effects in the presence of a major gene effect. Large sample sizes are required to demonstrate small but cumulative effects with regard to multiple genetic modifiers. It would be necessary to analyse many potential modifiers simultaneously to test for significance in different combinations of alleles at multiple candidate genes. Different degrees of linkage disequilibrium of the ACE I/D polymorphism with other potentially significant sequence changes in the gene and the modest degree of increased risk for CHD associated with the ACE DD

genotype, nevertheless argues against the likelihood that this marker is a clinically useful indicator of increased risk for CHD in Afrikaner FH patients.

In conclusion, this study demonstrates the potential value of mutation screening in populations with a certain enrichment of particular gene mutations and emphasises the need for comprehensive mutation screening and family studies in diagnostic settings. The importance of population-directed DNA screening strategies in FH diagnosis and in assessment of an individual's risk for the development of CHD has furthermore been highlighted. A comprehensive multilocus risk assessment strategy, including complete genotyping and accurate interpretation of genetic data, should in future be applied to determine an individual's risk for the development of CHD. Molecular analysis of patients with the FH phenotype may be considered a prerequisite for accurate diagnosis, genetic counselling and optimal treatment targeted at the cause of the disease.

7.4 FUTURE PERSPECTIVES

The importance of genetic testing in assessing prognosis and identifying high-risk subgroups is increasingly recognised and it can be expected that medical advances linked to the mapping of the human genome will radically change the healthcare process. Given the multifactorial nature of atherosclerotic disease, the discovery of new risk factors and causal pathways will lead to new avenues of attacking the heart disease epidemic in the 21st century. Future research aimed at redefining disease by genotype and disease mechanism may lead to targeted treatment and reduction of adverse side effects in patients that may not respond well to certain treatment strategies. This may be of particular relevance in LDLR-mutation-negative hypercholesterolaemics, who may carry mutations in novel genes implicated in ADH. Understanding the molecular basis of genetic predisposition to multifactorial diseases such as cardiovascular disease will depend on the analysis of specific sequence variants through association studies and genome scans to identify new candidate loci in regions detected through linkage analysis. Clinically informative population-directed subsets of disease markers may in future form the basis of panels for diagnostic or prognostic use in patient care.

The presence of multiple founder mutations in the South African populations raises the possibility that heterozygous FH may impose a selective advantage within the African context. Hobbs and colleagues (1990) acknowledged the possibility of a

Darwinian selection that may favour the heterozygous state in this part of the continent. It seems likely that such a selective agent may be related to infectious disease, since Netea and co-workers (1996) demonstrated that increased LDL plasma concentration might protect LDLR-deficient mice against lethal endotoxaemia and gram-negative infections. This was further substantiated by the finding that lipoproteins protect mice and rats against endotoxaemia (Rensen et al. 1997). Feingold and Grunefeld (1997) discussed the alterations that occur in lipid metabolism in response to infections and inflammation and the potential benefits that may be associated with these changes. Firstly, all lipoproteins bind and inactivate endotoxin (Hardardottir et al. 1994, 1995, Levine et al. 1993, Feingold et al. 1995) and in addition, also bind viruses and inhibit their infectivity (Hardarottir et al.1994, 1995). Secondly, elevated LDL levels may compete with viruses for receptors in cellular uptake and prevent further pathogenesis. Changes in triglyceride and HDL levels during infection may indirectly be involved in the immune response and tissue repair, while apolipoproteins neutralise viruses, including HIV (Owens et al. 1990). Fourthly, lipoproteins can target, bind and destruct parasites (Smith et al. 1995). Studies are underway to investigate our hypothesis that the high prevalence of FH in South Africa may not only be due to a founder effect, but may also be the consequence of a selective advantage within the African context. Preliminary data obtained in a case-control study raise the possibility that polymorphisms in the promoter region of the LDLR gene may be involved in disease progression of HIV-infected subjects (Scholtz et al. 2000b)

This study has emphasised the importance of a population-directed molecular screening strategy for the diagnosis of FH. Although the ethical and psychological consequences of genetic screening have to be considered, potential harmful effects of a molecular diagnosis of FH would be largely outweighed by the benefits of intensive treatment that may prevent or al least delay onset of CHD.

CHAPTER 8

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SUMMARY

Coronary heart disease (CHD) is one of the leading causes of mortality in Westernised countries and accounts for approximately 25% of all deaths. Elevated lipid and lipoprotein levels leading to atherosclerosis, affects about 4.8 million South Africans, placing them at high risk for developing CHD. Familial hypercholesterolaemia (FH) and familial defective apolipoprotein B-100 (FDB) are autosomal co-dominant inherited lipid disorders, the hallmark of these phenotypes being the elevation of plasma cholesterol concentrations and premature mortality from cardiovascular complications. Molecular defects in the low density lipoprotein receptor (LDLR) gene and apolipoprotein B (APOB) gene, respectively, underlie the phenotype in FH and FDB. DNA analysis of these loci, using a combination of three mutation screening strategies, was performed in the diverse South African population. No FDB-causing mutation was detected in any of the clinically defined hypercholesterolaemic patients included in the study, which underlines the low prevalence of this lipid disorder in the South African population.

A high sensitivity of extensive combined mutation screening strategies was demonstrated, since molecular lesions were detected in all Jewish hypercholesterolaemic patients analysed. Despite this, the gene defects could not be identified in all the clinically defined Afrikaner FH patients analysed. The disease phenotype in these patients may be caused by mutations in other genes underlying autosomal dominant hypercholesterolaemia (ADH).

The high prevalence of FH in several of the genetically distinct populations of South Africa, particularly the Afrikaners and Jews (~1/70), allows a population-based screening strategy. In addition to the three previously described Afrikaner founder mutations (D154N, D206E and V408M), two possibly minor founder mutations, D200G and S258L, were detected in Afrikaner FH homozygotes. Inclusion of these LDLR gene defects in routine DNA screening would improve the diagnostic service for FH. The Jewish founder mutation, 652delGGT, predominates in the majority (57%) of South African Jewish patients screened. This mutant allele became more prevalent in FH Jews of Lithuanian lineage (75%). LDLR gene mutation, N407K, identified in an Afrikaner-Jewish FH patient who is also heterozygous for mutation, D206E, appears to be associated with a mild FH phenotype. Follow-up family studies demonstrated that genotype-phenotype correlation studies are of utmost importance for the implementation of preventive treatment strategies.

Gender, age, race, home language, historical affiliation with specific religious groups, geographical distribution, as well as specific disease phenotypes may impart to the heterogeneous distribution of mutant alleles in a homogeneous population. The distribution of the three founder-related Afrikaner LDLR gene defects was shown to correlate significantly with home language (Afrikaans or English) and historical membership with the Gereformeerde Church. Analysis of the angiotensin-converting enzyme (ACE) gene insertion/deletion (I/D) polymorphism in the Afrikaner population supports the notion that age and the genetic background of population subgroups may contribute to the heterogeneity of this genetic marker. The potential for different degrees of linkage disequilibrium of the ACE I/D polymorphism with other potentially significant sequence changes in the gene and the modest degree of increased risk for CHD associated with the ACE DD genotype, argued against the likelihood of this marker being a clinically useful indicator of increased risk for CHD in Afrikaner FH patients.

This study highlights the potential prognostic value of DNA diagnosis in populations where a small number of mutations cause the disease in the vast majority of affected cases. The possible role of modifier genes in the phenotypic expression of FH has to be considered within a specific population context, since gene-environment interaction most likely contributed to the unique genetic make-up of the diverse South African population. A comprehensive multilocus risk assessment strategy, including complete genotyping and accurate interpretation of genetic data, should in future be applied to determine an individual's risk for the development of CHD. Molecular analysis of patients with the FH phenotype may be considered a prerequisite for accurate diagnosis, genetic counselling and optimal treatment targeted at the cause of the disease.

KEYWORDS: coronary heart disease, denaturing gradient gel electrophoresis, familial defective apolipoprotein B-100, familial hypercholesterolaemia, genotype-phenotype correlation studies, heteroduplex-single strand conformation polymorphism, low density lipoprotein receptor gene, modifier genes, molecular diagnosis, mutation screening, South African population

OPSOMMING

Koronêre hartvatsiekte (KHVS) is een van die hoofoorsake van mortaliteit in Westerse lande en dra by tot nagenoeg 25% van alle sterftes. Verhoogde lipied- en lipoproteïen vlakke lei tot aterosklerose en affekteer ongeveer 4.8 miljoen Suid-Afrikaners deur hulle in 'n hoë risikogroep vir die ontwikkeling van KHVS te plaas. Familiële hipercholesterolemie (FH) en familiële defektiewe apolipoproteïen B-100 (FDB) is outosomaal ko-dominante oorerflike lipiedsiektes, met verhoogde plasma cholesterol konsentrasies en premature sterftes vanweë kardiovaskulêre komplikasies as sleutelkenmerke van die fenotipe. Molekulêre defekte in die lae digtheid lipoproteïenreseptor (LDLR) geen en apolipoproteïen B (APOB) geen, onderskeidelik, is onderliggend aan die FH en FDB fenotipes. DNA analise van hierdie lokusse deur die gebruik van 'n kombinasie van drie mutasie-siftingstrategieë, is in die diverse Suid-Afrikaanse bevolking uitgevoer. Geen FDB-veroorsakende mutasies kon in enige van die klinies gedefinieërde hipercholesterolemie pasiënte wat ondersoek is, opgespoor word nie. Dit beklemtoon die lae voorkoms van hierdie lipiedsiekte in die Suid-Afrikaanse bevolking.

Die uitgebreide, gekombineerde mutasie-siftingstrategie het 'n hoë sensitiwiteit openbaar, aangesien mutasies by alle Joodse hipercholesterolemie pasiënte op wie analises uitgevoer is, aangetoon kon word. Daarenteen is mutasies nie by alle klinies gedefinieërde Afrikaner FH pasiënte wat ondersoek is, identifiseer nie. Die siektefenotipe by hierdie pasiënte kan moontlik veroorsaak word deur mutasies in ander gene wat by outosomale dominante hipercholesterolemie (ADH) betrokke is.

Die hoë voorkoms van FH in verskeie populasie groepe in Suid-Afrika, veral die Afrikaners en Jode (~1/70), leen homself tot 'n populasie-gerigte siftingstrategie. Bykomend tot die drie reeds beskryfde Afrikaner stigtersmutasies (D154N, D206E and V408M), is twee moontlike kleiner stigtersmutasies, D200G en S258L, in Afrikaner FH homosigote opgespoor. Insluiting van hierdie LDLR geendefekte by die roetine DNA siftingstrategie sal 'n verbeterde diagnostiese diens vir FH teweegbring. Die Joodse stigtersmutasie, 652delGGT, is teenwoordig in die meerderheid (57%) Suid-Afrikaanse Joodse pasiënte wat ondersoek is. Hierdie mutante alleel kom meer algemeen voor by Joodse FH pasiënte van Litauaanse afkoms (75%). Die LDLR geenmutasie, N407K, identifiseer in 'n Afrikaner-Joodse pasiënt wat ook heterosigoties vir mutasie, D206E, is, blyk geassosieerd te wees met 'n matige FH fenotipe. Opvolgondersoeke het getoon

dat genotipe-fenotipe korrelasie studies in families van uiterste belang is by die implementering van voorkomende behandeling-strategieë.

Geslag, ouderdom, ras, huistaal, geskiedkundige verwantskap aan spesifieke godsdienstige groepe, geografiese verspreiding, sowel as spesifieke siektefenotipes, mag almal deelagtig wees aan die heterogene verspreiding van mutante allele in 'n homogene bevolking. Die verspreiding van die drie LDLR Afrikaner stigtersmutasies korreleer statisties beduidend met huistaal (Afrikaans of Engels) en geskiedkundige lidmaatskap aan die Gereformeerde Kerk. Analise van die "angiotensin-converting enzyme" (ACE) geen invoeging/delesie (I/D) polimorfisme in die Afrikanerbevolking, staaf die gedagte dat ouderdom en genetiese agtergrond van bevolkingsubgroepe tot heterogeniteit van hierdie genetiese merker kan bydra. Die potensiaal van verskillende grade van koppelingsdisekwilibrium van die ACE polimorfisme met ander potensieël belangrike volgordeveranderings in die geen en die matige verhoogde risiko vir KHS wat met die ACE DD genotipe geassosieerd is, skakel die moontlikheid dat hierdie merker 'n klinies bruikbare indikator vir verhoogde KHS risiko is, grootliks uit.

Hierdie ondersoek benadruk die potensiële prognostiese waarde van DNA diagnose in bevolkings waar 'n klein aantal mutasies die siekte in die meerderheid geaffekteerde gevalle veroorsaak. Die moontlike rol van modifiserende gene by die fenotipiese uitdrukking van FH moet binne 'n spesifieke bevolkingskonteks beskou word, aangesien geen-omgewing interaksie baie waarskynlik 'n bydrae tot die unieke genetiese samestelling van die Suid-Afrikaanse bevolking gemaak het. 'n Uitgebreide multilokus risikobepalingstrategie wat volledige genotipering en akkurate interpretering van genetiese resultate insluit, behoort in die toekoms gebruik te word om 'n individu se risiko vir die ontwikkeling van KHVS te bepaal. Molekulêre analise van pasiënte met 'n FH fenotipe, kan as voorvereiste beskou word vir akkurate diagnose, genetiese raadgewing en optimale behandeling wat op die oorsaak van die siekte gerig is.

APPENDICES

APPENDIX 1

ELSEVIER

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ATHEROSCLEROSIS

MedPed FH: a paradigm for other common monogenic diseases in South Africa☆

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1. Introduction

Familial hypercholesterolemia (FH) is the most common autosomal dominant disease in South Africa. A molecular diagnostic service for FH was established in 1990, based on the finding that three low-density lipoprotein receptor LDL-receptor gene defects are responsible for the disease in up to 90% of affected Afrikaners. Subsequent studies have demonstrated that the common LDL-receptor gene mutations are also present in other ethnic groups, most likely due to multiple entries of disease genes into specific populations and/or founder effects coupled to geographic or cultural isolation. Increasing numbers of South African FH patients can therefore be characterized at the DNA level using a comprehensive population-directed DNA screening strategy.

2. Methods

Over recent years the MedPed approach has contributed largely to rapid patient identification and a growing awareness that early treatment of FH may prevent or at least substantially delay onset of cardiovascular disease.

3. Other applications

For the same reason that FH occurs at a high prevalence (~ 1/70) in Afrikaners, many other treatable single gene disorders are also common in this relatively homogeneous population of European decent. Consequently, at the Human Genetic Divisions of the Universities of Stellenbosch and the Orange Free State, we have extended the MedPed initiative to other common genetic diseases (Table 1) which meet the following criteria: (1) a single gene causing treatable/preventable serious illness; (2) validated diagnostic tests available; (3) some form of effective treatment or prevention available. With the exception of hereditary hemochromatosis (HH), all other genetic diseases incorporated in the South African MedPed approach are inherited in an autosomal dominant manner. The reason for including HH in this effort is because this iron-overloading disorder is considered to be the most common autosomal recessive disease in the Caucasian population worldwide, with an estimated prevalence of ~ 1/100 in South African Afrikaners. Recent studies have demonstrated that common HH-causing mutation (C282Y) occurs at a carrier frequency of 17% in South Africans of European decent, implying that genotyping of spouses may restrict investigation of children in families with genetic hemochromatosis. DNA diagnosis is particularly applicable to common genetic diseases like HH, which is potentially lethal, underdiagnosed, but treatable.

4. Results

In order to raise public awareness of the family-based genetic screening approach, we initiated a process of regular national MedPed meetings involving patients, physicians and geneticists. The first South African MedPed Genetic Information Day was held on the 2nd of July 1998, following the National Ge-

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Table 1
Treatable genetic diseases incorporated in the South African Medped initiative

Genetic disease	Description diagnosis	Clinical vs genetic	Treatment or prevention
FH: Familial hypercholesterolemia	Elevated LDL cholesterol, xanthomas and early heart attack deaths	High plasma cholesterol and/or xanthomas in patient and relatives vs direct mutation screening or haplotype analysis	Cholesterollowering drugs and diet can normalize cholesterol and prevent and/or substantially delay onset of cardiovascular disease
VP: Variegate porphyria or 'South African porphyria'	Acute porphyria caused by dominantly inherited gene, photosensitivity, abdominal pain, bulbar palsy, respiratory paralysis	Overproduction of porphyrins and their precursors in stool and urine, acute and cutaneous clinical features vs mutation screening	Avoid the sun, porphyrogenic drugs, beta-blockers, prophylaxis, hematin, glucose loading
FAP: Familial adenomatous polyposis coli	Dominantly inherited gene causing colon cancer, polyps	Occult FeHb+colonoscopy and biopsy vs mutation screening, haplotype analysis truncation test	Early detection and colonoscopy, prophylactic surgery can prevent fatal metastatic disease
HH: hereditary hemochromatosis	Common autosomal recessive disease causing heart disease, endocrine dysfunction due to iron overload damaging organs	High transferrin saturation and ferritin levels, liver biopsy vs mutation screening	Early diagnosis and regular phlebotomy minimise/prevent organ damage, iron chelators

netics Congress in Bloemfontein. Although only approximately 30 people attended the meeting, we believe that the event and the limited media coverage assisted local clinicians and families alike (Table 1) with respect to the importance of follow-up studies to trace defective genes. To date 374 individuals from the Bloemfontein region, mostly from the local lipid clinic, have been referred for genetic analysis of FH. Mutations D154N, D206E or V408M have been identified in 80 patients, who were advised on cholesterol-lowering treatment by their clinicians. Five patients were homozygous or compound heterozygous for mutations D206E and/or V408M.

5. Conclusion

We believe that the MedPed approach via early diagnosis and proper treatment will lead to prevention of many premature deaths in South Africa. The aim of our effort is to translate fundamental advances in genetics for the benefit of clinical practice.

APPENDIX 2

Nonradioactive Multiplex PCR Screening Strategy for the Simultaneous Detection of Multiple Low-density Lipoprotein Receptor Gene Mutations

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We have developed a rapid, nonradioactive screening test enabling the simultaneous analysis of three low-density lipoprotein receptor (LDLR) gene mutations (D154N, D206E, and V408M), which together account for familial hypercholesterolemia (FH) in ~90% of the South African Afrikaner population. The assay is designed so that FH patients, negative for these founder-related mutations (found in descendants of European settlers), subsequently can be screened for unknown mutations in the mutation-rich exon 4 of the LDLR gene. Our screening assay consists of two steps: (1) multiplex allele-specific PCR amplification of exons 4 and 9, and (2) simultaneous analysis of single- and double-strand conformational polymorphisms in exon 4 by vertical electrophoresis on low cross-linked polyacrylamide gels. The simplicity, specificity, and versatility of the multiplex assay makes it an ideal system for routine screening of FH mutations in large population samples.

The mutational heterogeneity of familial hypercholesterolemia (FH), a common autosomal dominant disease caused by mutations in the low-density lipoprotein receptor (LDLR) gene,[1] complicates disease diagnosis at the DNA level. A molecular diagnosis of FH was shown to be more accurate than a clinical diagnosis.[2,3] Also, because it may be of more prognostic value,[4] much interest is focused on cost-effective methods of screening for disease-related LDLR gene mutations.

The single-strand conformation polymorphism (SSCP) technique, described by Orita et al.,[5] is used most commonly worldwide to screen for mutations. It has also been used to detect the majority of LDLR gene mutations identified to date.[6] This simple and convenient general screening method also is being used for the molecular diagnosis of FH in heterogeneous populations.[7,8] Mutation-specific screening methods have been developed for the molecular diagnosis of FH in more homogeneous populations, where a small number of mutations account for the disease in the majority of cases.[9–12] The increased prevalence of FH in Afrikaners (~1/80) is caused by three founder-related point mutations in the LDLR gene that most likely originated in Europe,[6,8,13,14] and are also present in South Africans of mixed race.[15]

In this study we describe the development of an assay for the simultaneous analysis of the three founder-related Afrikaner mutations in a single PCR. With this convenient multiplex amplification refractory mutation system (ARMS)–PCR, the use of expensive restriction enzymes, radioactivity, and time-consuming, allele-specific oligonucleotide hybridization methods[9,16] can be avoided. Furthermore, the assay was designed in such a manner that PCR products obtained from samples provided by hypercholesterolemics without the common mutations can be screened subsequently for unknown mutations in the mutation-rich exon 4[6] of the LDLR gene. Recently, we have demonstrated that low cross-linked polyacrylamide gels supplemented with 15% urea are highly sensitive in detecting such single-base substitutions as heteroduplexes.[17] We have modified these conditions slightly for simultaneous analysis of SSCPs on the same gels. It has been suggested that a combination of heteroduplex and SSCP analyses should bring mutation detection closer to 100%.[18,19]

MATERIALS AND METHODS

Genomic DNA

Genomic DNA was extracted from blood samples of normal controls and FH patients heterozygous for previously described LDLR gene defects.[9–11,20,21] The DNA was PCR-amplified and used to standardize mutation detection of the founder-related FH Afrikaner-1 (D206E), -2 (V408M), and -3 (D154N) mutations[9] by ARMS-PCR,[22] and also to establish conditions suitable for simultaneous

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analysis of six known mutations (Table 1) in the 3′ half of exon 4 by SSCP[5] and heteroduplex formation[18] on a single polyacrylamide gel.

Multiplex PCR

Three common and three ARMS primers of the LDLR gene,[23] specific for the three mutant alleles, were used in the multiplex PCR: COMM 1, 5′-CGAGGCCTCCTGCCCGGTGCTCACC-3′; COMM 2, 5′-GCTCACCTGCAGATCATTCTCTGGG-3′; COMM 3, 5′-GGGACCCAGGGACAGGTGATAGGAC-3′; ARMS 1, 5′-CCCGCCCATACCGCAGTTTTCCTCC-3′; ARMS 2, 5′-AGCCTCATCCCCAACCTGAGGACCA-3′; and ARMS 3, 5′-GGGCCTGCGACAACGACCCCGACTGCGAAA-3′. All reactions were performed in a volume of 50 μl containing ~0.5 μg of genomic DNA; 2 units of *Taq* DNA polymerase (Boehringer Mannheim); 1x *Taq* DNA polymerase buffer (supplied by Boehringer Mannheim); 200 μM each dATP, dCTP, dGTP, and dTTP (disodium salt, Boehringer Mannheim); 25 pmoles of primer COMM 1; 50 pmoles of primer ARMS 1; 10 pmoles of primers COMM 2 and ARMS 2; 100 pmoles of primers ARMS 3 and COMM 3; 2 mM tetramethylammonium chloride (Me_4NCl); and 15% glycerol. Reaction mixtures were overlaid with light mineral oil (Sigma, 50 μl) and subjected to DNA denaturation at 94°C for 5 min, and then to two amplification cycles: (1) 15 cycles at 94°C for 1 min, 62°C for 1 min, 72°C for 2 min; and (2) 20 cycles at 94°C for 1 min, 55°C for 1 min, 72°C for 2 min. PCR products were electrophoresed for 3 hr in 3% Metaphor gels (FMC Bioproducts) or for 2 hr at 100 V in 10% polyacrylamide gels, and stained with ethidium bromide.

Heteroduplex–SSCP Analyses

DNA fragments of 330 bp comprising the 3′-half of exon 4 of the LDLR gene (the internal control fragment in the multiplex PCR) and spanning six different mutation sites were PCR–amplified according to Kotze et al.[9] Ten microliters of each PCR product was mixed with an equal volume of gel loading buffer (95% formamide, 20 mM EDTA, and 0.05% bromophenol blue, 0.05% xylene cyanol), and denatured at 95°C for 2 min and immediately placed on ice. The samples were loaded directly onto 30-cm vertical and 1-mm-thick 10% polyacrylamide gels with 1% cross-linking (%C, ratio of the percent concentration of *N,N′*-methylenebisacrylamide to the concentration of total acrylamide monomer) and run overnight at room temperature at 250 V.[17] Gels were supplemented with 15% and 7.5% urea (Stratagene), respectively, and stained in a solution of 0.6× TBE containing 1 μg/ml of ethidium bromide.

RESULTS

Multiplex PCR Assay

A dual-purpose multiplex ARMS–PCR assay (see Fig. 1) was developed to identify FH heterozygotes with the FH-1 (D206E), -2 (V408M), or -3 (D154N) mutations, and to subject those without these mutations to a further heteroduplex–SSCP screen of exon 4 of the LDLR gene. The photo shows the allelic differentiation obtained directly after PCR amplification and agarose gel electrophoresis of genomic DNA from individuals heterozygous for the FH-1, -2, and -3 mutations, respectively. With a DNA sample from a control subject, a 330-bp product was derived from the internal control primers only (COMM 1 and COMM 3). When DNA samples of FH-1, -2, and -3 heterozygotes were used in the PCR reaction, the expected 285-, 100-, and 262-bp fragments, respectively, were generated. The presence of the 330-bp exon 4 fragment (internal control) in all tubes indicated that amplification occurred in all of the reactions. The remaining internal control PCR products of hypercholesterolemics without the three known mutations subsequently can be subjected to heteroduplex and SSCP analysis in polyacrylamide gels, to screen for other mutations in exon 4 of the LDLR gene.

TABLE 1 Mutations in Exon 4 of the LDLR Gene Analyzed by Heteroduplex–SSCP Analyses of PCR-amplified DNA

Codon change	Nucleotide change	Reference
Single-base substitutions		
D154N	G_{523}-A	9
D206E	C_{681}-G	9
E207K	G_{682}-A	10
Small rearrangements		
Deletion 197	delete 3 bp after 651	11
Insertion 206	insert 18 bp after 681	20
Deletion 168	delete 23 bp after 567	21

Heteroduplex–SSCP

DNA samples from six FH patients heterozygous for different mutations in exon 4 of the LDLR gene (Table 1) were PCR-amplified and examined simultaneously by heteroduplex and SSCP analyses on the same polyacrylamide gel after ethidium bromide staining. Electrophoresis at room temperature in 1% C polyacrylamide gels supplemented with 15% urea resulted in heteroduplex detection of PCR products in five samples from FH patients. They were heterozygous for an 18-bp insertion, a 23-bp deletion, a 3-bp deletion, a C → G-base change at codon 206 (D206E), and a G → A-base change at codon 207 (E207K), respectively. However, none of these mutations could be detected by SSCP analysis (data not shown). Lowering the urea concentration to 7.5% did not affect the number of mutations detectable by heteroduplex analysis. However, with the lowering of the urea concentration, three of the mutations (one of which was not detected by heteroduplex analysis) could be detected by SSCP. The combined screening approach thus enabled detection of all six mutations by heteroduplex (18-bp insertion, 23-bp deletion, 3–bp deletion, D206E, E207K) and/or SSCP (18-bp insertion, 23-bp deletion, D154N) analysis on a single polyacrylamide gel (Fig. 2).

PCR-amplified DNA, spanning the three point mutations analyzed by heteroduplex–SSCP, was loaded onto both sides of the same gel shown in Figure 2, to test the reproducibility of the bands obtained in 1% C polyacrylamide gels supplemented with 7.5% urea. Although the heteroduplexes resulting from mutations D206E (lanes 4,8) and E207K (lanes

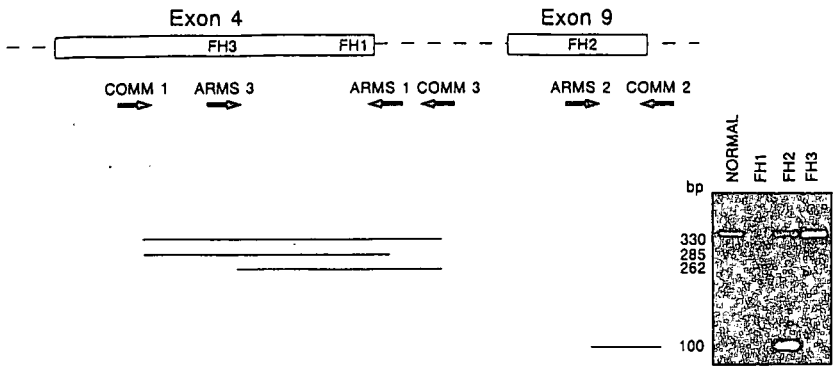

FIGURE 1 Analysis of the FH Afrikaner-1 (D206E), -2 (V408M), and -3 (D154N) mutations by multiplex PCR and gel electrophoresis. The relative positions of the three mutations, and the allele-specific (ARMS) primers and common (COMM) primers are indicated on the partial map of the LDLR gene. (*Left* to *right*) The four lanes in the agarose gel contain amplified DNA from individuals without the three mutations, heterozygous for the FH1 mutation, heterozygous for the FH2 mutation, and heterozygous for the FH3 mutation, respectively. The sizes of the amplification products are shown in base pairs (bp).

2,10) could be observed in all four lanes, the bands are less clear on the right side of the gel. This phenomenon probably can be ascribed to impurities in the gel. Similar smearing, which interferes with the analysis, was observed when the same gel mix was used for several weeks. The aberrant SSCP pattern generated by mutation D154N, as characterized by an additional band between the two normal alleles, is shown in Figure 2, lanes 3 and 9. Although the same amount of PCR product was loaded in both lanes, less double-stranded DNA is observed in lane 3 compared with lane 9. A smaller proportion of the DNA loaded in lane 3 probably renatured after the denaturation step, resulting in visualization of additional single-strand conformations in this lane.

DISCUSSION

The multiplex PCR assay described in this study provides a rapid and reliable method for routine screening of FH-related mutations in large population samples, provided suitable precautions are taken to circumvent possible mistypings. Detection of the desired PCR products and specificity of the reaction were found to be largely dependent on the annealing temperature used. Furthermore, different primer sets (and also different batches of the same primer sets) were amplified with slightly different efficiencies. The largest, 330-bp fragment of exon 4 was amplified preferentially when annealing temperatures ranged between 60°C and 68°C, resulting in failure to amplify the 285- and/or 262-bp fragments (or in the presence of very faint bands). This competition priming was reduced by lowering the annealing temperature to 55°C after the first 15 cycles. Separate amplification of the various primer sets has shown that the 330-bp internal control PCR fragment was not obtained at this temperature, whereas the smaller exon 4 fragments specific for the FH-1 (D206E) and FH-3 (D154N) mutations were amplified effectively (data not shown). The addition of glycerol in the PCR[24] was found to further enhance the amplification of the 285-bp FH-1 mutation-specific fragment, whereas the addition of an excess of the ARMS 3 and COMM 3 primers (and limiting the COMM 1 primer) improved the yield of the 262-bp fragment in FH heterozygotes with the FH-3 mutation. Standardization of the multiplex assay was complicated by failure to obtain this 262-bp fragment at an intensity comparable to that of the other bands. For this reason, the length of the ARMS 3 primer was increased to 30 nucleotides compared with the 25-nucleotide lengths of all of the other primers. Use of a 20-nucleotide ARMS 3 primer (5'-CAACGACCCCGACTGCGAAA-3') resulted in complete failure to amplify the expected 252-bp fragment in the multiplex PCR (data not shown). Thus, it is clear that primer length can be varied to increase or decrease selectively the yield of specific PCR products during multiplex reactions. Amplification of the 100-bp fragment specific for the FH-2 mutation (V408M) in exon 9 occurred independently of the above reactions, and the specificity of the reaction was increased by the introduction of an additional mismatch near the 3' end of primer ARMS 2 to avoid false-positive results.[15] We also included tetramethylammonium chloride (Me_4NCl) in the multiplex PCR reaction, because it eliminates the preferential melting point of AT versus GC base pairs. It also reduces the presence of nonspecific fragments caused by mispriming.[25]

The multiplex ARMS–PCR can detect FH patients heterozygous for one or two of the Afrikaner mutations but cannot distinguish heterozygotes from true ho-

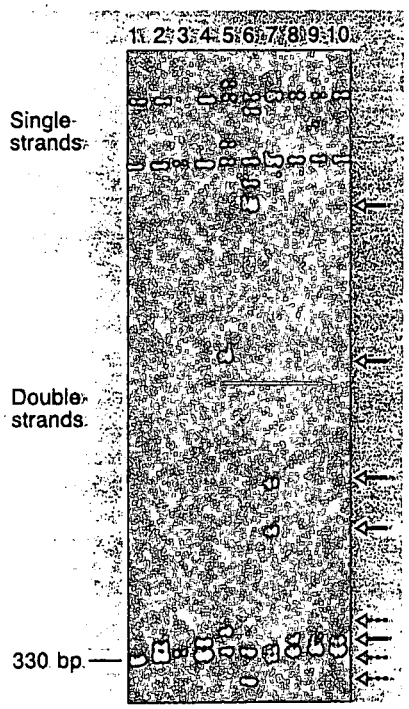

FIGURE 2 Heteroduplex–SSCP analyses of the six known mutations in the LDLR gene. PCR products of 330 bp were electrophoresed on 10% (1% C) polyacrylamide gels supplemented with 7.5% urea, and were subsequently stained with ethidium bromide. (Lane *1*) PCR-amplified DNA from a mutation-negative control; (lanes *2,10*) PCR products from FH patients heterozygous for mutations E207K; (lanes *3,9*) D154N; (lanes *4,8*) D206E; (lane *5*) an 18-bp insertion at codon 206; (lane *6*) A 23-bp deletion at codon 168; and (lane *7*) A 3-bp deletion at codon 197 were analyzed. Homoduplexes (broken arrows) and heteroduplexes (solid arrows) observed in double-stranded DNA are indicated on the right side of the gel.

mozygotes with two identical LDLR gene mutations. Subsequent restriction enzyme analysis of PCR-products can allow an accurate molecular diagnosis of these relatively rare cases.[9] Because an unequivocal clinical diagnosis of FH usually can be made in homozygotes, we focused the molecular diagnosis of FH on heterozygotes whose clinical diagnoses may be complicated by variability in phenotypic expression.[2–4] This screening approach scaled down the cost of a molecular diagnosis of FH in Afrikaners[26] to one-fifth the amount needed previously for restriction enzyme and/or oligonucleotide hybridization analysis.[9]

Knowledge that exon 4 of the LDLR gene is particularly mutation-rich and appears to be prone to various types of mutational events[6–8] was implemented in our screening strategy. The 330-bp internal control fragment comprising the 3′ half of exon 4 was specifically coamplified in the multiplex PCR to avoid false-negative results in the ARMS assay and also to subsequently screen this PCR product for FH-related mutations other than the three founder-related Afrikaner mutations. About 20% of LDLR mutations occur in this region of the gene.[6] DNA samples from patients heterozygous for six different mutations in exon 4 were used to establish conditions suitable for heteroduplex and SSCP analyses on a single polyacrylamide gel. Electrophoresis of denatured PCR products, under conditions that were previously shown to be highly sensitive in detecting single-base substitutions as heteroduplexes in undenatured DNA,[17] indicated that the urea concentration of 15% in the low cross-linked polyacrylamide gels is too high for SSCP analysis of the exon 4 LDLR gene fragments. The lowering of the urea concentration to 7.5% allowed mutation detection by both SSCP and heteroduplex analysis, because sufficient reannealing of DNA strands occurred to simultaneously allow the analysis of heteroduplex formation in the faster-migrating, double-stranded DNA on the same polyacrylamide gel.

All of the mutations analyzed could be detected, thus illustrating that a combination of SSCP and heteroduplex analyses is highly sensitive in detecting small mutations. Failure to detect the small 3-bp deletion by SSCP indicated that the relatively large size of the exon 4 fragment being analyzed influences the likelihood of detecting mutations in PCR-amplified DNA negatively, as has been shown previously by others.[27] The 3-bp deletion at codon 197 could readily be detected as an SSCP in a smaller PCR product of 237 bp (data not shown). Heteroduplex formation does not appear to be equally sensitive to the size of the PCR product analyzed and can be detected in relatively large PCR fragments by proportionally increasing electrophoresis times.[28,29]

Although it remains to be investigated as to how valuable combined heteroduplex–SSCP analyses are in improving detection of small mutations, this method nevertheless allows optimal use of the mutation-rich exon 4 PCR product being obtained in the multiplex PCR in patients without the three founder-related Afrikaner mutations. It also has the advantage of being technically straightforward and can be carried out without the use of isotopes because the DNA is stained with ethidium bromide.[30] Furthermore, including the analysis of the faster-migrating, double-stranded DNA in the screening approach gives an indication of the type of mutation involved. A mutant homoduplex resulting from an insertion in PCR-amplified DNA usually migrates more slowly on polyacrylamide gels than the normal double-stranded PCR product, whereas a mutant homoduplex resulting from a deletion usually migrates faster. An exception to this general pattern was, however, recently observed in the adenomatous polyposis coli gene, where the mutant homoduplex band resulting from an AT base pair insertion migrated faster than the normal homoduplexes in the unaffected individuals.[31] Heteroduplex bands were invariably found to migrate slower than homoduplex bands.

A further advantage of our multiplex screening strategy is that other mutation-specific ARMS primers can be included in the PCR for mutation detection in other exons or other genes, provided that different-sized DNA fragments are generated. For example, inclusion of the ARMS primers described by Wenham et al.[32] for detection of the apolipoprotein (apo) B_{3500} mutation,[33] causing familial defective apo B-100 (FDB),[34] generates the expected 187-bp ARMS product in FDB heterozygotes (data not shown). This allows differential molecular diagnoses of FH and FDB,[35] which share similar clinical characteristics. Because primers specific for mutations not prevalent in the study population similarly can be excluded from the PCR, the multiplex screening strategy described in this study is versatile and should be useful in various population groups.

The recent demonstration that mutational heterogeneity in the LDLR gene influences the phenotypic expression of FH[4] has emphasized the need for rapid PCR assays that may assist in the assessment of an individual's risk for the development of coronary heart disease (CHD). Mutation detection by multiplex ARMS–PCR, followed by heteroduplex–SSCP analyses in subjects without founder-related mutations, is an effective screening method for known and new LDLR gene mutations. This screening approach recently has resulted in the identification of the first molecularly characterized de novo mutation in exon 4 of the LDLR gene.[20] Conditions suitable for the detection of the six known mutations in exon 4 of the LDLR gene by heteroduplex and SSCP analyses were determined and can now be applied to other genes or other regions in the LDLR gene to screen various populations for mutations underlying genetic disease. Our data also provide evidence in favor of the hypothesis that addition of mildly denaturing solvents (urea in this case) can amplify the tendency of mismatched bases to produce conformational changes and thereby increase the differential migration of normal and mutant fragments during gel electrophoresis.[5,29,36]

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