Use of DNA haplotype analysis in diagnosis of familial hypercholesterolaemia in 31 German families

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Abstract

In the majority of patients, familial hypercholesterolaemia (FH) is caused by different mutations affecting the well defined low density lipoprotein receptor (LDLR) gene. However, 3% of patients in Munich with a clinical diagnosis of FH have a particular mutation in the apolipoprotein B gene causing familial defective apolipoprotein B-100 (FDB). To date none of the LDLR mutations causing FH in German patients has been identified and it is therefore not yet possible to use DNA technology for direct diagnosis. However, indirect molecular diagnosis based on genetic linkage of common restriction fragment length polymorphisms (RFLPs) of the LDLR gene can be used for diagnosis in family studies. Patients with FDB were excluded from this study. Genotypes and haplotypes of four RFLPs (*StuI*, *ApaI* 5', *PvuII*, and *NcoI*) were determined in a total of 164 independent alleles from 31 pedigrees that included 222 subjects. Allele frequencies and four RFLP haplotype distribution did not differ significantly from those reported in other populations. The applicability of DNA diagnosis in our lipid clinic is comparable with the estimation from calculations on frequencies and heterozygosities of RFLPs, as predicted using these four RFLPs before. On the basis of cosegregation of haplotypes and normo- or hypercholesterolaemia in one or more sibs or offspring, defective and normal LDLR gene alleles could be distinguished in 42 of 58 heterozygous FH patients who were heterozygous for at least one RFLP. In 97 of

134 (72%) children of these 42 subjects, diagnosis of FH could be carried out unambiguously. Cholesterol levels of subjects with normal and defective LDLR genes show significant overlap, especially in younger cases, and FH was diagnosed in 10 cases with normal cholesterol levels and excluded in five cases with slightly raised cholesterol levels.

Familial hypercholesterolaemia (FH) is a common inherited disease characterised clinically by raised concentrations of low density lipoprotein (LDL) cholesterol levels in the blood, tendon xanthomata, an increased risk of myocardial infarction, and genetically by autosomal dominant inheritance.¹ Brown, Goldstein, and coworkers have shown over the last few years that FH results from different genetic defects in a cell surface receptor that normally controls the removal of LDL from plasma.¹⁻³ Some populations, like the Afrikaners in South Africa,⁴ the French Canadians,⁵ and the Finnish,⁶ carry only a few mutations. In these cases, direct molecular diagnosis using appropriate DNA technology is possible if the specific LDLR defect is known. No mutation has yet been identified in the German population. We have previously shown, using four RFLPs of the LDLR gene, that there are eight different haplotypes carrying defective alleles of the gene in a sample of FH patients from Munich.⁷ This suggests at least eight different mutations must be present and because of the mutational heterogeneity only indirect molecular diagnosis of FH is possible based on genetic linkage analysis of common DNA polymorphisms of the LDLR gene in family studies.

Today, at least 10 RFLPs of the human LDLR gene are known.⁸ For some of these RFLPs the allele frequencies are too low to be used for linkage analysis, and linkage disequilibrium among RFLPs reduces their informativeness in pedigree analysis. Recently, a strategy for haplotype analysis of the LDLR gene was suggested in a Caucasian American sample, which identified the RFLPs that were most useful.⁹

We have used four RFLPs (*StuI*, *ApaI* 5', *PvuII*, and *NcoI*) to carry out diagnosis of FH in families

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attending a lipid clinic. Allele frequencies and haplotype data were compared with data reported from other populations. The number of informative subjects for these four RFLPs was compared with predictive data based on pairwise polymorphism information content (PIC) values¹⁰ and index of heterozygosity calculated on genotype and haplotype distribution. Cholesterol levels of subjects with normal and defective LDLRs were also compared.

Patients and methods

PATIENTS

We have investigated 222 subjects from 31 German families attending a lipid clinic in Munich, in which at least five family members were available for family studies. In these families, 58 subjects had 134 children in whom diagnosis should be carried out by recombinant DNA techniques. In 21 of these families FH was confirmed by skin fibroblast studies as described previously.¹¹

Blood samples were taken 12 hours after the last meal. Cholesterol levels were determined after a period without drug treatment of at least six weeks or when the patients came to the lipid clinic for the first time and before therapy. HDL cholesterol was determined by a precipitation method and LDL cholesterol was estimated by the formula of Friedewald *et al.*¹² if triglycerides were below 200 mg/dl. Lipoproteins were measured by preparative ultracentrifugation, according to Havel *et al.*,¹³ in all other cases.

PROBES FOR THE LDL RECEPTOR GENE

The gene probe used was pLDLR-2HH1, a kind gift of Dr D W Russell, Dallas, USA. The plasmid contains a 1.9 kb fragment of the full length cDNA which hybridises with exon 11 to exon 18 of the gene. The fragment was excised from a 1% low gelling temperature agarose gel and labelled by means of a random oligonucleotide primer with ³²P-dCTP at 3000 Ci/mmol, as described previously.⁷

DNA ANALYSIS

Total genomic DNA was extracted from white blood cells using a triton X-100 lysis method.¹⁴ A total of 5 µg of high molecular weight genomic DNA was digested with the restriction enzymes *Apa*II and *Bam*HI, *Nco*I, *Pvu*II, and *Stu*I (Amersham International) using conditions recommended by the manufacturer. The fragments were separated by electrophoresis on 0.8% agarose gels. After electrophoresis, the DNA was transferred from the gel to nylon membranes (Hybond N, Amersham International) and fixed with UV light radiation. The

membranes hybridised with the radioactive LDLR gene probe in 7% sodium dodecyl sulphate (SDS) and 0.5 mol/l phosphate buffer (pH 7.0) at 65°C using a rotation oven (Bachhofer, West Germany) for 24 hours. Filters were washed finally in 1 × SSC (150 mmol/l NaCl, 15 mmol/l sodium citrate), 0.1% SDS and exposed to x ray film (Kodak X-Omat AR) for one to three days. Familial defective apo B-100 (FDB) owing to the 3500 mutation was excluded in all index cases using allele specific oligomelting, as described previously.¹⁵

Results

A total of 222 subjects was genotyped for the *Stu*I, *Apa*II 5', *Pvu*II, and *Nco*I RFLPs. Allele frequencies were determined by gene counting. For the unrelated subjects, the genotype distribution for all RFLPs was not significantly different from the expected value if the population is in Hardy-Weinberg equilibrium (data not shown).

The frequencies and polymorphism of these four RFLPs were determined among 164 independent LDLR gene alleles (table 1). To assess diagnosis with these RFLPs in our population, the number of heterozygous subjects was compared with a standardised estimate by calculating the PIC values for the polymorphisms.¹⁰ The index of heterozygosity for *Stu*I, *Apa*II 5', and *Nco*I was slightly higher than expected from PIC values, whereas the index of heterozygosity for *Pvu*II was slightly lower. Table 2 shows the cumulative heterozygosity of the four RFLPs. For *Apa*II 5', 50% of our probands were heterozygous and therefore informative for DNA diagnosis. With the additional use of *Nco*I, the heterozygosity index increases to a cumulative value

Table 1 Frequencies and PIC values of four RFLPs at the LDLR gene locus among 164 independent chromosomes compared to heterozygosity in 58 heterozygous FH patients.

RFLP	Frequency of + site	PIC value	Heterozygosity
<i>Stu</i> I	0.911	0.095	0.138 (n = 8/58)
<i>Apa</i> II 5'	0.472	0.374	0.500 (n = 29/58)
<i>Pvu</i> II	0.350	0.352	0.345 (n = 20/58)
<i>Nco</i> I	0.706	0.329	0.483 (n = 28/58)

Table 2 Cumulative heterozygosity of four RFLPs at the LDLR gene locus among 58 heterozygous FH patients.

RFLP	Location of site	Cumulative heterozygosity
<i>Apa</i> II 5'	Intron 15	0.500 (n = 29/58)
<i>Nco</i> I	Exon 18	0.672 (n = 39/58)
<i>Pvu</i> II	Intron 15	0.672 (n = 39/58)
<i>Stu</i> I	Exon 8	0.724 (n = 42/58)

of 0.67. As predicted by calculation of linkage disequilibrium with *NcoI*^{19,17} the addition of the *PvuII* genotype gives no further information. The inclusion of *StuI* slightly increases the index of heterozygosity to 72%. In conclusion, the *ApaII* 5' and *NcoI* RFLPs are the most informative and should be chosen first for family studies. The additional use of *PvuII* and *StuI* is of limited value because of high linkage disequilibrium and low allele frequency, respectively.

DNA haplotypes for all subjects were constructed by segregation analysis based on the assumption that there has been no recombination events within these families. Haplotypes of 131 normal and 33 defective independent LDLR gene alleles are shown in table 3. In comparison with data from a Caucasian American population⁹ and a group of patients from South Africa,¹⁶ there was no significant difference in haplotype distribution in normal subjects. In the Afrikaner population a well defined founder effect causes a significant difference in haplotype distribution between the normal population and FH subjects.⁴ Haplotype distribution was not significantly different between normal and defective alleles in the Munich population, but haplotype 3 is more common in the FH group. This haplotype may therefore carry the most frequent mutant allele in the Munich population.

In the 31 pedigrees, there were 58 parents who had a diagnosis of FH based on plasma cholesterol levels above the 95th centile for age and gender. In 42 of these 58 subjects the defective and normal alleles of the LDLR gene could be distinguished on

the basis of cosegregation between haplotypes and normo- or hypercholesterolaemia in one or more sibs or offspring. To illustrate how genetic diagnosis of FH was carried out using haplotype analysis, a representative family pedigree is shown in fig 1A in which the haplotype 11* of the proband cosegregates with hypercholesterolaemia. Therefore, the defective allele can be identified as 11* in this particular pedigree. Fig 1B shows another family in which the allele 8* cosegregates with hypercholesterolaemia. However, in this family the proband has inherited the defective allele but does not show the FH phenotype. In 97 of 133 children confirmation of the genetic diagnosis of FH could be carried out. In 60 cases the child had inherited the allele of the LDLR gene confirmed to be defective in the kindred and in 37 cases the normal allele had been inherited (fig 2). Table 4 shows the lipid levels of both groups. There was a significant difference ($p < 0.001$) in mean cholesterol and LDL cholesterol between the groups using standard analysis of variance, but there was overlap in LDL cholesterol levels between the groups. Fig 3 shows age and sex dependent total cholesterol levels of family members with defective and normal LDLR genes. In 10 cases (three females, seven males), diagnosis of FH was confirmed based on inheritance of the defective allele in the kindred although cholesterol levels were below the 95th centile for this sample.¹⁸ In five cases (three females, two males), the defective allele in the kindred had not been inherited and a genetic diagnosis of FH was excluded, although cholesterol levels were above the 95th centile.

Table 3 Haplotype distribution of 131 normal and 33 defective independent LDL receptor genes for four RFLPs from 31 families including 222 subjects from a German population in comparison to other populations. (+) denotes the presence of the variable restriction site and (–) the absence of the site. Difference in haplotype distribution is not significant in the German population. Data for Caucasian Americans reported by Leitersdorf et al⁹ and for white Afrikaners by Kotze et al.¹⁶

Haplotype	StuI	ApaII	PvuI	NcoI	Germany				USA		South Africa			
					Normal		FH		Normal		Normal		FH	
					No	%	No	%	No	%	No	%	No	%
1	+	+	+	+	3	2.3			1	0.8	1	0.9		
2	+	+	+	—										
3	+	+	—	+	35	26.5	17	51.7	59	48.0	39	35.2	2	2.0
4	+	+	—	—	10	7.6			3	2.4	6	5.4	2	2.0
5	+	—	+	+	26	19.9	8	24.2	20	16.3	26	23.4		
6	+	—	+	—	1	0.8			1	0.8				
7	+	—	—	+	8	6.1	2	6.0	2	1.6	2	1.8	85	85.9
8	+	—	—	—	33	25.2	5	15.1	30	24.4	31	27.9		
9	—	+	+	+										
10	—	+	+	—	1	0.8								
11	—	+	—	+	1	0.8	1	3.0						
12	—	+	—	—	1	0.8								
13	—	—	+	+	7	5.3			7	5.7	6	5.4	10	10.1
14	—	—	+	—	1	0.8								
15	—	—	—	+	3	2.3								
16	—	—	—	—	1	0.8								
Total					131		33		123		111		99

Figure 1 Pedigrees of two representative FH families. The arrow indicates the proband. Total serum cholesterol levels are given in mg/dl. Hypercholesterolaemia above the 95th centile for age and gender¹⁸ is shown by shaded symbols. The genotypes of four RFLP haplotypes are outlined according to table 3. (A) Family 1454. The allele 11* characterised by its four RFLP haplotype cosegregates with the FH phenotype. (B) Family 1317. The allele 8* cosegregates with the FH phenotype. However, in this family the proband aged 6 has inherited the defective allele and exhibits cholesterol levels below the 95th centile for age and gender.

Discussion

The aim of this study was to determine the applicability of DNA diagnosis with RFLPs of the LDLR gene in 31 German FH families. Since the cloning of the human LDLR gene by Brown, Goldstein, and coworkers, FH can be diagnosed by recombinant DNA technology either using restriction enzyme mapping to detect major rearrangements of the gene or using RFLPs and linkage analysis in family studies. To date, at least 20 deletions or insertions have been mapped with restriction enzyme digests. These defects concern all four classes of mutations that were already known before cloning of the LDLR gene and disrupt the synthesis, intracellular transport, binding, and internalisation function of

Figure 2 Flow chart showing the applicability of DNA diagnosis in FH using haplotype analysis in the LDLR gene in 31 German families.

the LDLR gene. In most populations, major rearrangements account for only a small number of defects. However, two deletions have been found that are most common in two different populations. In a French-Canadian population a 10 kb deletion can be identified in 60% of FH subjects⁵ and in a Finnish population a 9.5 kb deletion can be identified in 50% of FH patients. In the Munich population, major rearrangements of the LDLR gene are rare (unpublished data) and therefore restriction enzyme mapping is not feasible for diagnosis in most families.

In cases in which unknown point mutations cause FH, diagnosis can be carried out using RFLPs and linkage analysis in family studies. The first polymorphism in the LDLR gene and its use in diagnosis was described in 1985.¹⁹ Since then at least 10 RFLPs have been described and their applicability has been shown by various authors. In general, the use of RFLPs for diagnosis is determined by their degree of heterogeneity and linkage to other RFLPs. The genotype distribution, the degree of heterogeneity, and the linkage disequilibrium is now known for several polymorphisms in different populations^{7,16,17,20} and a strategy for haplotype analysis of the LDLR gene was recently presented from data from a normal Caucasian American population.⁹ However, less is known about the general applicability of linkage analysis in family studies to diagnose FH. Using linkage analysis with three RFLP haplotypes (*PvuII*, *ApaII*, and *AvaII*) it was possible to identify the haplotype of the affected gene in 57

Table 4 Mean (SE) lipid levels in 97 family members in which diagnosis of FH has been confirmed or excluded using four RFLPs at the LDLR gene locus. Total cholesterol and LDL cholesterol levels are significantly different between FH and normal subjects only.

Group	Sex	No	Age	Trgl	Chol	LDL	HDL
LDLR defective	Males	35	34 (2.6)	152 (21.0)	326* (13.2)	251† (12.5)	44 (2.2)
	Females	25	29 (3.2)	77 (5.7)	317* (15.4)	241† (15.2)	60 (3.9)
LDLR normal	Males	29	28 (3.0)	143 (28.8)	204* (6.7)	130† (4.2)	49 (2.8)
	Females	8	27 (6.0)	97 (19.7)	203* (10.7)	130† (12.6)	54 (3.1)

*F = 78.241, df = 1, p < 0.00001. †F = 85.981, df = 1, p < 0.00001.

Italian families and to carry out unequivocal diagnosis in 67% of the cases.²⁰

In our German population, comparison of haplotype distribution showed no striking difference between the normal Caucasian American and normal African population from which comparable data are available. Within our population there is also no striking difference in haplotype distribution between normal and affected genes. Therefore, it is not possible to diagnose FH by identifying a certain haplotype in our population. This approach has been shown to be useful in an African population only.¹⁶ However, using linkage analysis with four

polymorphisms we were able to identify the affected gene in 31 German FH families and diagnosis of FH was feasible in 72% of family members. These results are in very good agreement with the estimation from calculations on frequencies and heterozygosities of RFLPs as predicted before.⁹ Comparing data from pedigree analysis with lipid levels, we identified subjects with normal cholesterol levels and defective LDLR genes and subjects with moderate hypercholesterolaemia and normal LDLR genes. It is a well known phenomenon that lipid levels do overlap between subjects with normal and defective LDL receptors, especially in younger

Figure 3 Age dependent cholesterol levels of (A) female and (B) male FH heterozygotes and normal family members in 31 families.

cases, and even within the same family. Similar results have been shown in patients with FH confirmed by tissue culture experiments.²¹ Cholesterol levels do not always allow unequivocal diagnosis, especially in children and young adults, since these values may fall in the upper range of normal.²² As appropriate diet and specific treatment of patients with FH can reduce their risk of coronary heart disease, it is advantageous to identify affected subjects before the onset of clinical signs and symptoms.

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