Recurrent LDL-receptor mutation causes familial hypercholesterolaemia in South African coloureds and Afrikaners

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Three low-density lipoprotein receptor (LDLR) gene mutations were previously shown to cause familial hypercholesterolaemia (FH) in up to 90% of affected Afrikaners. Association of each mutation with a single chromosomal background provided molecular genetic evidence that the proposed 'founder gene effect' was responsible for the high prevalence of FH among white Afrikaners. In this study we report the identification of the FH Afrikaner-2 (FH2) mutation, Val_{408} to Met, in the so-called coloured population of South Africa, a people of mixed ancestry, with rapid non-radioactive methods for mutation detection. Haplotype analysis with polymorphisms on both sides of the FH2 mutation indicated that the identical LDLR gene mutations found in two different South African population groups were caused by independent events at a potential CpG mutational 'hot spot'. The allelic variation giving rise to the different chromosomal backgrounds of the FH2 mutation does not affect the properties of the abnormal LDLR protein product which causes FH in these subjects. This mutation is thus expected to cause the same severe form of FH in affected coloureds as was previously demonstrated in Afrikaners. Detection of mutant LDLR gene alleles in polymerase chain reaction products, directly after gel electrophoresis, now allows accurate presymptomatic diagnosis of the FH2 mutation in FH patients from two different South African population groups.

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Familial hypercholesterolaemia (FH) contributes significantly to the high mortality from coronary heart disease (CHD) in several South African populations. Since more than 150 different naturally occurring low-density lipoprotein receptor (LDLR) gene mutations have been described to date,[1] direct molecular diagnosis of FH is currently limited to genetically homogeneous populations, where the majority of individuals carry only a few disease related mutations. In such populations, like the South African Afrikaners[2-4] and Ashkenazi Jews,[5] specific founder gene defects can be diagnosed accurately. In the Afrikaner population it has been shown that three LDLR gene mutations cause FH in approximately 90% of affected individuals.[6,7] Although each founder-related mutation was found to be associated with a single chromosomal background in the Afrikaner patients analysed,[4,8] subsequent screening of Dutch FH patients for these mutations revealed a second haplotype associated with the FH Afrikaner-2 (FH2) mutation, Val_{408} to Met.[9] In this study we identified the FH2 mutation on a third haplotype in two unrelated individuals of mixed ancestry, using direct non-radioactive molecular methods that are more convenient than those previously described, which involve the use of radioactivity and/or expensive restriction enzymes.[6,7]

Materials and methods

Analysis of the FH Afrikaner-2 mutation

DNA samples of individuals, previously typed by hybridisation with allele-specific oligonucleotides for the absence or presence of the FH2 mutation (Val_{408} to Met, G to A mutation at base 1285 in exon 9 of the LDLR gene)[6] were used to standardise detection of the mutation with the amplification refractory mutation system (ARMS)[10] and single-strand conformation polymorphism (SSCP) method.[11] SSCP analysis was performed on polymerase chain reaction (PCR) products amplified with exon 9-specific oligonucleotides N2 (5'-GCTCCATCGCCTACCTCTTC-3') and A2 (5'-GCTCACCTGCAGATCATTCTCTGGG-3') as described previously.[2] Aliquots of the 172 bp amplified products (5 µ1) were denatured with alkali (0,5M NaOH, 10 mM EDTA) at 42°C for 5 minutes, electrophoresed at 80 V overnight at room temperature in a non-denaturing 10% polyacrylamide gel supplemented with 5% glycerol, and stained with ethidium bromide (0,5 µg ml^{-1}) for 10 minutes. Reactions for the ARMS analysis were performed in two separate tubes per sample, each containing one allele-specific oligonucleotide, 9.6 (5'-AGCCTCATCCCCAACCTGAGGACCG-3', normal allele) or 9.7 (5'-AGCCTCATCCCCAACCTGAGGACCA-3', mutant allele), in conjunction with a common distal primer A2 (see above). The underlined base was deliberately destabilised to ensure allele-specificity. Primers specific for exon 26 of the apolipoprotein (apo) B gene,[12] AB-1 (5'-GGAGCAGTTGACCACAAGCTTAGC-3') and AB-2 (5'-CAGGGTGGCTTTGCTTGTATGTTC-3'), were included in all reactions and served to provide an internal control PCR product of 345 bp. Approximately 0,5 µg genomic DNA were used in the amplification reaction, with 5 pmol of each primer and one unit Taq DNA polymerase in a volume of 50 µl. Taq DNA polymerase, exclusively from Boehringer Mannheim, was used in the ARMS assay, since unacceptable nonspecific bands were obtained when amplification was performed with Taq polymerase purchased from some other manufacturers. The four deoxynucleoside triphosphates were each added to 100 µM in 1 x Taq DNA polymerase buffer (Boehringer Mannheim). Reaction mixtures were overlaid with light mineral oil (Sigma, 50 µl), subjected to DNA denaturation at 94°C for 5 minutes and then to 30 cycles of amplification (93°C for 1 minute; 60°C for 1 minute; 72°C for 2 minutes). PCR products were analysed in 2% agarose gels.

Subjects

The ARMS method described above was used to identify the FH2 mutation in 2 coloured and 28 Afrikaner FH patients unrelated to the second degree. A total of 24 family members were also screened for the FH2 mutation with either the ARMS or SSCP method. Patients attended the lipid clinic at Tygerberg Hospital, or were referred for DNA analysis of FH from other lipid clinics in South Africa, general practitioners or the Department of National Health and Population Development.

Haplotype analysis

Haplotypic arrangements at 14 polymorphic sites within or closely linked to the LDLR gene were analysed: the ApaLI restriction fragment length polymorphism (RFLP) in intron 3,[13] the TaqI RFLP in intron 4,[14] the SphI RFLP in intron 6,[15] the SmaI RFLP in intron 7,[16] the StuI RFLP in exon 8,[17] the guanine to adenine base change in exon 10,[18] the HincII RFLP in exon 12,[19] the BstEII RFLP in intron 12,[20,21] the AvaII RFLP in exon 13,[22] the PvuII RFLP in intron 15,[23] the NcoI RFLP in exon 18,[24] the two MspI RFLPs in exon 18,[25] and the 3'-flanking PstI RFLP.[26] Genotypes at the polymorphic sites in introns 4 and 7, and exons 8, 10, 12, 13 and 18 were determined by PCR-based methods, while the others were analysed by Southern blot analysis.

FH-associated haplotypes for all subjects were deduced by segregation analysis, based on the assumption that there were no recombination events within families, or by homozygosity for the polymorphisms analysed.

Results

Fig. 1 shows the allelic differentiation obtained with the ARMS and SSCP methods respectively, directly after PCR amplification and gel electrophoresis of genomic DNA from normal individuals, heterozygotes and homozygotes for the FH2 mutation of the LDLR gene. When the ARMS method was used on the DNA of a normal individual, a product was derived only from the internal control primers and primers 9.6 and A2 (Fig. 1A, lane 1). No LDLR gene product (or very faint bands) was observed when primer 9.7, specific for the mutant allele, replaced primer 9.6 (lane 2). When DNA of a FH2 heterozygote was used in the two separate reactions, the expected 100 bp fragment was generated when either primer 9.6 or 9.7 was included in the reaction (Fig. 1A, lanes 3 and 4). Amplification occurred with DNA from a FH2 homozygote only when the mutant oligonucleotide was used (lane 6). Presence of the 345 bp apo B fragment (internal control) in all tubes indicated that amplification occurred in all the reactions. SSCP analysis was performed on the same

set of DNA samples, as well as on some additional samples, to test the mutation-detection efficiency of this method for the FH2 mutation. In Fig. 1B the G alleles in normal individuals could readily be distinguished from the A alleles in affected individuals.

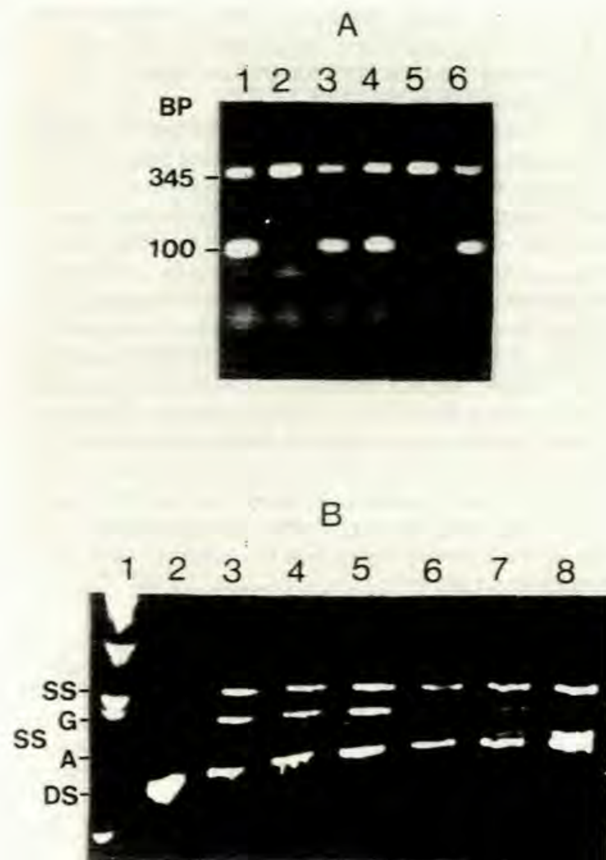

Fig. 1. Analysis of the FH2 mutation after PCR amplification and gel electrophoresis. (A) ARMS analysis of a normal control (lanes 1 - 2), a FH heterozygote (lanes 3 - 4) and a FH homozygote (lanes 5 - 6). ARMS primers were used as follows: lanes 1, 3 and 5, normal G-allele (9.6); lanes 2, 4 and 6, mutant A-allele (9.7). These were used in conjunction with a common distal primer (A2). Primers AB-1 and AB-2, producing PCR products of 345 bp, were used in each reaction as internal controls. PCR products were electrophoresed in 2% agarose gels. (B) Non-isotopic SSCP analysis of alkali-denatured PCR products of 172 bp on 10% acrylamide gels containing 5% glycerol. Lanes: (1) 1 kb DNA ladder, (2) undenatured DNA control, (3 - 5) DNA from normal controls, (6 - 7) DNA from FH patients heterozygous for the FH2 mutation and (8) DNA from a FH homozygote. (bp = base pairs; ss = single-stranded DNA; ds = double-stranded DNA.)

The above methods were used to identify 30 FH2 heterozygotes during an ongoing screening of South African patients for known LDLR gene mutations. Subsequent genotyping at 14 polymorphic sites showed that the FH2-associated haplotype in two unrelated coloured families differs from that in Afrikaners at 5 polymorphic sites framing the FH2 mutation. Table I compares the different polymorphic alleles associated with the FH2 mutation in the Afrikaner and coloured populations. None of the South African FH2 heterozygotes studied showed disease-association with the second haplotype described in Dutch patients.[9]

Table I. LDLR gene haplotypes associated with the FH2 mutation in two different South African population groups

Polymorphism	Location	Afrikaner	Mixed race
ApaLI	Intron 3	+	+
TaqI	Intron 4	–	–
SphI	Intron 6	+	+
SmaI	Intron 7	–	+
StuI	Exon 8	+	+
—	Exon 10	A	A
HincII	Exon 12	+	+
BstEII	Intron 12	–	–
AvaII	Exon 13	–	–
PvuII	Intron 15	–	+
NcoI	Exon 18	–	+
MspI	Exon 18	–	+
MspI	Exon 18	–	+
PstI	3' flanking	–	–

+ = presence of a restriction enzyme cutting site; – = absence of a restriction enzyme cutting site; A = adenine; G = guanine.

Discussion

Three founder-related LDLR gene mutations increased the prevalence of FH among Afrikaners to about 1 in 80,[27] compared with 1 in 500 in most other population groups. One of these, the so-called FH2 mutation, causes a severe phenotype[28] and accounts for FH in about 20% of Afrikaners.[6] In this study we applied non-radioactive ARMS and/or SSCP methods to screen for the FH2 mutation in hypercholesterolaemics who were referred to us for a DNA diagnosis of FH.[29] By utilising the exon 9-specific PCR amplification primers, described previously, for analysis of the FH2 mutation by differential oligonucleotide hybridisation,[6] we obtained reproducible results by direct visualisation of different alleles after electrophoresis in SSCP gels and ethidium bromide staining.[30] Direct analysis of the FH2 mutation, following allele-specific amplification by the ARMS method and gel electrophoresis, required synthesis of primers specific for the mutant and normal alleles for use in two separate PCR reactions. When screening FH heterozygotes we found a single PCR reaction, using only the mutant ARMS primers, to be adequate. The specificity of the ARMS primers was increased by the introduction of an additional mismatch near the 3' ends to avoid false-positive results. False-negative results were excluded by the co-amplification of a 345 bp PCR product of the apo B gene. The apo B gene products obtained in the reactions can be used simultaneously to screen hypercholesterolaemics for point mutations that cause familial defective apolipoprotein B-100,[31] by heteroduplex analysis in low cross-linking polyacrylamide gels.[32]

Haplotype analysis in the normolipidaemic and FH populations has previously shown the FH2 mutation to be associated with a single 10-RFLP arrangement in Afrikaners.[8] Subsequent screening for the 3 Afrikaner founder LDLR gene mutations in patients of Dutch descent resulted in the identification of the FH2 mutation in 1,6% of subjects.[9] Of the 16 FH2 heterozygotes detected in the Netherlands, 7 shared a LDLR gene haplotype with Afrikaner patients. The remaining 9 patients had the same FH2

mutation-associated haplotype, which was partly identical to that in Afrikaners and could have arisen from a single recombinational event. Genealogical and extended haplotype studies support the hypothesis that the FH2 mutation originated in the Netherlands and was introduced into South Africa by an early Dutch settler in the 17th century (J. C. Defesche — personal communication and unpublished results).

In this study the FH2 mutation was identified in 30 South African patients who had not previously been analysed in respect of the haplotype on which the mutation occurred.[8] To investigate the relationship between the FH2 mutations detected in both Afrikaner and coloured patients, we performed haplotype analysis using 14 polymorphic sites at the LDLR locus.[13-26] As expected, the chromosomal background originally described for the FH2 mutation[8] showed an association with the mutation in the Afrikaner patients, but a different disease-associated haplotype was identified in two unrelated coloured patients. These haplotypes differ on both sides of the mutation in exon 9: at the PvuII, NcoI and two MspI RFLPs on the 3' side and at the SmaI RFLP on the 5' side.

The above results suggest that independent mutational events in exon 9 of the LDLR gene gave rise to the FH2 mutation in two different South African populations. The fact that the single-base substitution in exon 9 involves a methylated CpG dinucleotide,[33] known to mutate frequently,[34] further supports this theory. The FH2 mutation could, however, also have had a common origin if the chromosomal background were changed by a crossover between the FH2 mutation in exon 9 and the PvuII polymorphism in intron 15, and a base pair change occurred at the SmaI site. This possible, but improbable, mechanism was excluded by haplotype analyses of normolipidaemic coloureds (data not shown). Since the heterozygosity of most RFLPs described earlier at the 5' end of the LDLR gene is very low,[13,14] conclusive evidence against the second mechanism's giving rise to the different FH2 mutation-associated haplotypes came from an analysis of the recently-described SmaI RFLP in intron 7.[16] The frequency of this excellent LDLR gene haplotype marker is high in FH and normal individuals from both population groups (data not shown). These results provide evidence against the possibility that the base pair change that creates the SmaI site in the 'coloured' haplotype arose on an ancient FH2 mutation-associated haplotype that underwent a single recombination event.

The historic ethnic origin of the FH2 mutation found in coloureds was not investigated further. Whites, Asians and blacks have contributed to the gene pool of the present-day coloured population of South Africa.[35] We do believe that, as more FH patients in more population groups both in and outside South Africa are genotyped at the disease locus, further light will be shed on the origins of this mutation.

Although the properties of the abnormal LDLR are not affected by the polymorphic sites that allow differentiation of the chromosomal background for the FH2 mutation in the two South African patient groups, the presence of a PvuII site in coloureds may result in a more favourable genetic background for the phenotypic expression of FH. This neutral PvuII RFLP in intron 15, that is probably in linkage disequilibrium with a functionally important sequence change in the LDLR gene, shows significant association with cholesterol level variation in healthy individuals.[36,37] The effect associated with the PvuII rare allele (+) is to lower total and LDL-cholesterol levels, and its relatively higher frequency in individuals over the age of 65 years suggests that the allele may be associated with increased fitness for survival.[37] Variability in the clinical expression of FH, due to the influence of other genetic and non-genetic factors, has previously been illustrated in South African patients with identical mutant genes.[38,39]

Our findings confirm the previous assessment of a single FH2 founder gene among Afrikaners,[2,4,8] most probably introduced into South Africa by a single individual of Dutch origin. Screening for the FH2 mutation and its associated haplotypes in other South African population groups has shown that this mutation is also common in people of mixed racial ancestry as a result of an independent mutational event. To date, 4 coloured FH2 heterozygotes have been identified, and in all of them the newly-described haplotype was associated with the disease (A. D. Marais — personal communication). Both non-radioactive methods described in this study facilitate rapid, inexpensive diagnosis of the FH2 gene mutation in hyperlipidaemic individuals. Previous observations that this specific mutation causes considerably elevated cholesterol levels and death at a young age underline the potential importance of detecting FH2 patients presymptomatically.[9,28,39]

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