

The identification of two low-density lipoprotein receptor gene mutations in South African familial hypercholesterolaemia

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Summary

Two point mutations were discovered in the low-density lipoprotein genes of patients with familial hypercholesterolaemia (FH). Defective genes were cloned and/or amplified by the polymerase chain reaction (PCR) method and the DNA sequences determined. A guanine to adenine base transition in exon 4 was found to be the molecular defect in 20% of cases of FH in the Afrikaner population. A second mutation, a guanine to adenine base substitution in exon 9, was identified in two homozygous FH individuals. Restriction enzyme analysis of PCR-amplified DNA from blood and tissue samples now permits accurate diagnosis of these mutations.

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Familial hypercholesterolaemia (FH) is an autosomal dominant disease caused by mutations in the low-density lipoprotein receptor (LDL-R) gene located on chromosome 19.¹ The defective receptor causes LDL to accumulate to high levels in plasma, which eventually leads to atherosclerosis and premature heart attacks.

The prevalence of the disease is very high (1 in 80) in the white Afrikaans-speaking section of the South African population.² A founder gene effect has been suggested to explain the high frequency of FH in this so-called Afrikaner population.³ The Afrikaner has indeed a history of a small founder community, which remained isolated through religious belief and cultural bonds. We recently reported on the segregation of genetic markers in and around the LDL-R gene in the FH and unaffected Afrikaner populations. Evidence was presented that at least two founders must have been responsible for the high frequency of FH in this population group.⁴ Haplotype studies showed that a presumed defective gene co-segregated with the rare allele of a *Nco* I restriction fragment length polymorphism (RFLP)⁵ in 70% of FH families. A second haplotype, determined by the rare allele of the *Stu* I enzyme,⁶ co-segregated with a second gene defect in 20% of FH families. This association was confirmed in genetic marker studies in 27 FH homozygotes.⁷

To date the diagnosis of FH in South Africa relied on the measurement of total or LDL cholesterol levels. It is known

that an elevated level of LDL cholesterol is not always an accurate indication of the disease, since overlapping values are found in affected and unaffected individuals.

We have now cloned the defective genes and by DNA sequence analysis two different single basepair (bp) mutations have been identified. Amplification of genomic DNA from blood and tissue samples by the polymerase chain reaction (PCR)⁸ method, using synthetic oligonucleotides specific for the mutated exons, now permits accurate diagnosis of these mutations after restriction enzyme digestion and electrophoresis of the DNA.

Material and methods

Patients and families

Blood samples were obtained from the FH patients, their families and normocholesterolaemic individuals, as previously described.^{4,7}

Genomic cloning

A genomic library was constructed in lambda L47.1 using *Bgl* II digested DNA from an FH patient who was known to have the rare allele of *Stu* I haplotype. A recombinant clone (FH8-30) was isolated from the library using a 1,05 kb *Pst* I subclone of pLDLR-3, a full-length cDNA clone, as a probe.⁷

Restriction endonuclease mapping and Southern blotting indicated that the 15 kb *Bgl* II clone FH8-30 contained exons 4-11 of the LDL-R gene. The insert also lacked the *Stu* I binding site in exon 8. A 7 kb *Hind* III/*Bam* HI fragment of the clone was further subcloned into pBR 328 and used for DNA sequencing.

DNA amplification

Genomic and cloned DNA amplification using Taq polymerase (Amersham) was performed according to the procedure described by Saiki *et al.*⁹ The following oligonucleotide primers (Beckman Instruments) were used for amplification of specific exons of the LDL-R gene:

Exon 4: 5' end — 5'-CATCCATCCCTGCAGCCCC-3' (H)
3' end — 5'-CCATACCGCAGTTTTCTCG-3' (I)
Exon 9: 5' end — 5'-GCTCCATCGCTACCTCTTC-3'
3' end — 5'-CTGCAGATCATTCTGGGA-3'

The PCR products were used for either direct DNA sequencing or enzyme digestion and electrophoresis.

DNA sequencing

DNA fragments were sequenced by the dideoxy chain-termination method¹⁰ using the oligonucleotide primers specific for the exons as described above. Both strands were sequenced (Taq Track, Promega). The results were compared with the normal sequence.¹¹

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Restriction digests of amplified DNA

Aliquots (50 μ l) of amplified DNA sequences were digested with the specific restriction enzyme and electrophoresed in 2% agarose gels. The DNA fragments were stained with ethidium bromide and visualised by ultraviolet fluorescence.

Results

Single base substitution in exon 4

A single base substitution at position 523 was observed in the DNA sequence of exon 4 of the LDL-R gene cloned from an FH patient in whom the rare allele of the *Stu* I polymorphism co-segregates with the disease. In Fig. 1A the guanine to adenine transition is marked in the partial autoradiogram.

This mutation was observed by comparing the cloned sequences with those found in a normal allele. The existence of this mutation was further confirmed in DNA sequence analysis of 3 additional FH patients who carry the rare *Stu* I allele as a marker.

Restriction enzyme digests

From the DNA sequence data of the normal and mutated exon 4 and the known binding sites of the available restriction enzymes, we deduced that there would be a loss of an *Mbo* II enzyme binding site in DNA of individuals with the mutated allele. DNA from a normal individual, the cloned fragment (FH8-30) and a *Stu* I-associated FH patient was amplified by PCR and digested with *Mbo* II (Fig. 2). DNA from the

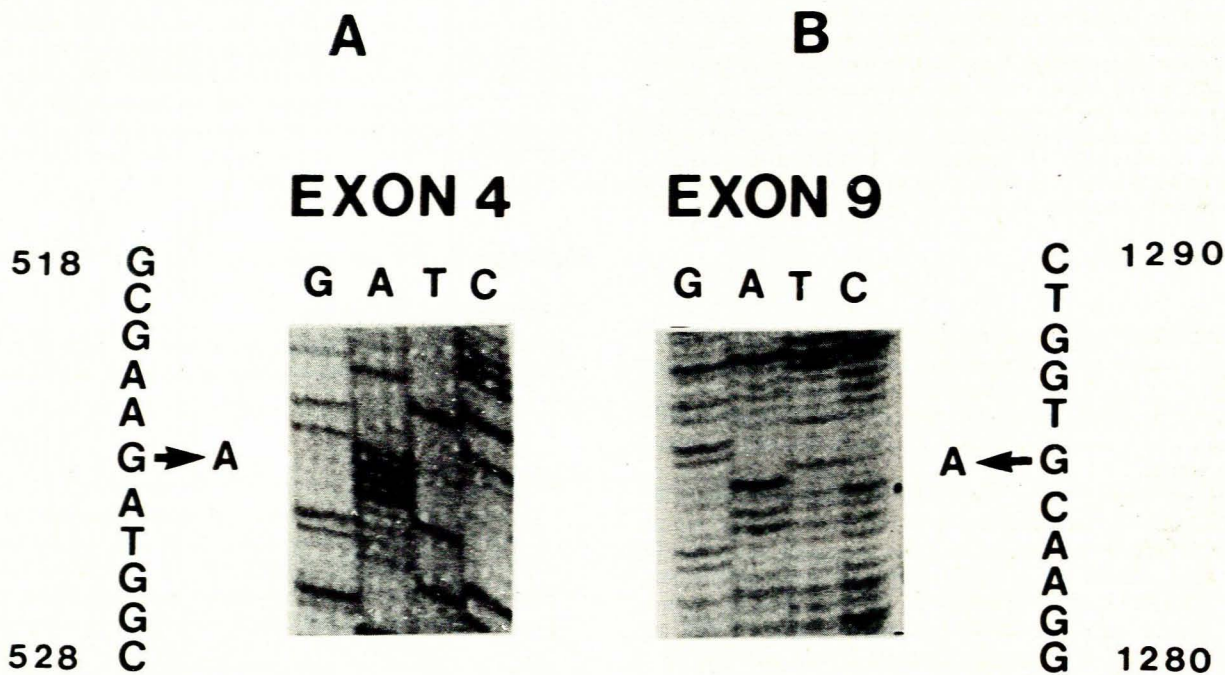


Fig. 1. Autoradiograms of sequencing gels demonstrating point mutations in the LDL-R gene of Afrikaner FH patients. A. The mutant sequence in exon 4 of a *Stu* I-associated allele. A guanine to adenine transition (G — A) is indicated at nucleotide position 523. B. The mutant sequence of an *Nco* I-associated FH homozygote. A single base change from guanine to adenine is indicated at nucleotide position 1285.

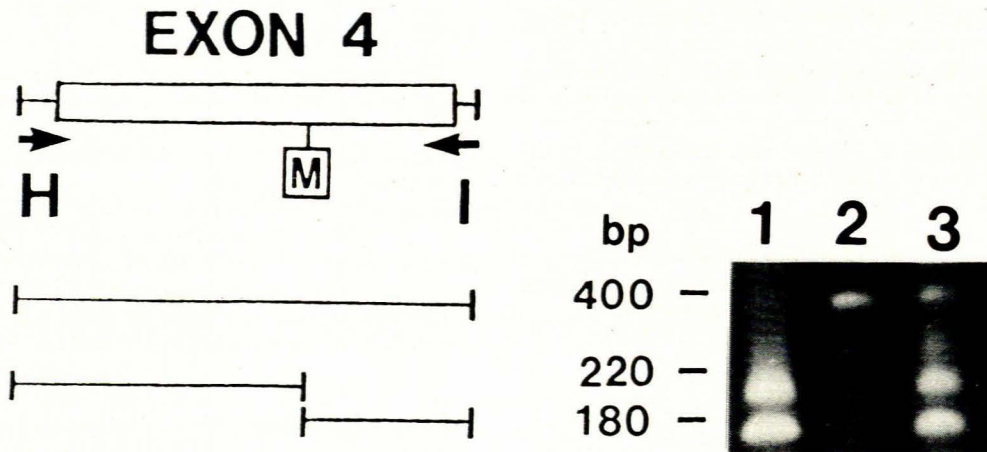


Fig. 2. Gel electrophoresis of *Mbo* II-digested PCR-amplified exon 4 DNA. The diagram shows the mutation in exon 4 and the expected fragment sizes after *Mbo* II digestion. Lane 1: DNA from a normal control individual. Lane 2: A cloned fragment from the rare *Stu* I allele. Lane 3: DNA from a *Stu* I-associated FH heterozygote.

normal individual was digested to completion, and fragments of 220 and 180 bp were observed. The digestion of DNA from the cloned fragment resulted in one fragment of 400 bp indicating loss of the *Mbo* II binding site (lane 2). DNA from a FH heterozygote showed heterozygosity after digestion by *Mbo* II (lane 3). This confirms that the enzyme did not digest the DNA of the mutated allele, while that of the normal allele was digested.

Analysis of the exon 4 mutation in normal and FH families

The presence of the exon 4 mutation, using PCR-amplified DNA and restriction digestion with *Mbo* II, was studied in 2 informative FH families of whom 10 members were FH heterozygotes with the associated rare *Stu* I allele. The presence of the mutation, as seen by the absence of the *Mbo* II restriction site in the amplified DNA of affected heterozygotes, correlates with high LDL cholesterol levels in these individuals. This is a further confirmation that the exon 4 mutation does indeed cause the disease in these families.

The absence of the mutation in 20 normal individuals, of whom 6 were also found to have the associated rare *Stu* I allele, further substantiates this finding.

Single base substitution in exon 9

A single base substitution at position 1285 was observed in the DNA sequence of exon 9 from amplified DNA of 2 FH homozygotes, in whom the rare allele of the *Nco* I RFLP cosegregates with the disease. In Fig. 1B the guanine to adenine transition is marked in the partial autoradiogram. The mutation was observed by comparing the sequence data of exon 9 of both alleles of two homozygotes with the sequence data from normal controls.

From the sequence data of both the normal and the mutated allele of exon 9, we deduced that there would be a loss of the restriction enzyme binding site of the enzyme *Mae* II in individuals with the mutated allele. Using the oligonucleotides for exon 9 as primers, the amplified DNA from true homozygotes would not digest using the enzyme *Mae* II. The heterozygotes would show a similar heterozygosity in fragment sizes as was illustrated for exon 4 mutation. The *Mae* II enzyme was not readily available for further studies prior to publication.

Discussion

DNA from a compound homozygote was cloned and the *Stu* I-associated allele of the LDL-R gene was isolated from the library for further characterisation. A point mutation was observed in exon 4 of this allele, causing loss of an *Mbo* II binding site. Evidence that the mutation causes the disease in these families was demonstrated in studies where the mutation was seen to segregate with high LDL cholesterol levels. Preliminary data indicate that this mutation occurs in all *Stu* I-associated FH patients. Extrapolation of the haplotype data indicates that the exon 4 mutation will account for about 20% of the founder-related defect in the Afrikaner population.

In 2 FH homozygotes with the associated *Nco* I allele a point mutation was found in exon 9. This mutation resulted in the loss of an *Mae* II binding site. Population screening for the incidence of this mutation in FH patients will reveal whether all the *Nco* I-associated FH patients have the same exon 9 mutation of the gene.

Accurate diagnosis of these mutations is now possible by *Mbo* II and *Mae* II digestion of PCR-amplified DNA.

This study has shown that direct molecular diagnosis of these gene mutations in familial hypercholesterolaemia is simple, reliable and allows for rapid unequivocal diagnosis of the disease. Identification of the defective genes in heterozygous parents also permits the prenatal diagnosis of homozygosity in chorionic or amniotic tissue of the fetus and offers the possibility for termination of the pregnancy.

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