CYS127S (FH-Kairouan) and D245N (FH-Tozeur) mutations in the LDL receptor gene in Tunisian families with familial hypercholesterolaemia

M N Slimane, S Lestavel, V Clavey, F Maatouk, M H Ben Fahrat, J C Fruchart, M Hammami, P Benlian

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Familial hypercholesterolaemia (FH, MIM 143890) is a dominantly inherited metabolic disorder caused by various mutations of the LDL receptor (LDL-R) gene causing delayed clearance of serum low density lipoproteins (LDL). The prevalence of homozygous FH has been estimated to be 1 in a million in European and North American populations. In central and southern Tunisia, the prevalence of homozygous FH is approximately seven-fold higher. The minimal estimated frequency of heterozygotes is about 1/160. These frequencies are as high as those found in populations with a high degree of inbreeding, such as Afrikaners in South Africa, Christian Lebanese, Finns, and French Canadians. In these populations, only a limited number of LDL-R gene mutations cause FH in the majority of affected cases as the result of a founder effect. Because of numerous consanguineous unions in rural areas in Tunisia, it can be predicted that the number of mutations causing FH may be limited. Moreover, we had reported that heterozygous FH might be misdiagnosed through classical symptom assessment because of mild clinical expression. Therefore, Tunisian patients with FH would be easier to identify by direct genotyping of frequent disease causing mutations. We have recently identified one founder frameshift mutation (F472; del TCT, ins AGAGACA, →43 aa-term) in five families originating from the coastal region of Monastir in Tunisia.

The aim of this study was to characterise further the spectrum of LDL receptor gene mutations in the Tunisian population. In order to investigate the influence of potential modifier alleles on phenotypic expression of FH, apoE polymorphism and common LPL gene variants were analysed. Here we describe two novel missense mutations in the LDL-R gene causing FH in two unrelated families from central and southern Tunisia.

MATERIALS AND METHODS

Patients

Probands were defined by at least one person (aged <30 years) presenting with multiple planar, tuberous, or tendinous xanthomas and plasma LDL cholesterol >400 mg/dl. Family members, who had given informed consent for the study, were examined for the presence of extravascular deposits (xanthomas or corneal arcus), cardiovascular risk factors, and signs of cardiovascular disease. Plasma lipids (TC, TG) and HDL cholesterol were measured by enzymatic and precipitation methods as previously described. LDL cholesterol was calculated by the Friedewald formula. According to these criteria, FH was identified in two unrelated families: family A included nine subjects from the Kairouan area (central Tunisia) and family B included six subjects from the Tozeur area (southern Tunisia).

Key points

- The objective of the study was to characterise further the spectrum of LDL receptor (LDL-R) gene mutations causing familial hypercholesterolaemia (FH) in central and southern Tunisia.
- DNA sequencing of the promoter and exonic regions of the LDL-R gene was performed in 15 clinically diagnosed FH patients from two unrelated families attending the lipid clinic in Monastir. Segregation analysis was performed in relatives. Functional studies of LDL-R activity on primary cell cultures from skin fibroblasts were undertaken.
- Two missense mutations in the LDL receptor gene were identified. One is a novel mutation replacing G with C at nucleotide 443 (443 G>C) causing a substitution of cysteine for serine at codon 127 in exon 4. This mutation, named C127S or FH-Kairouan, creates a recognition site for MnlI and was detected in three homozygotes and six heterozygotes from the same family. The second mutation replaces G with A at nucleotide 796 (796 G>A) and causes a substitution of aspartic acid for asparagine at codon 245 in exon 5. This mutation, named D245N or (FH-Tozeur) and involving a CpG dinucleotide, has been reported in a Dutch patient. In the homozygotes, both mutations were associated with a severe clinical expression and abolished LDL receptor activity in cultured fibroblasts obtained from skin biopsy. Heterozygous carriers of either of the two mutations had significantly increased plasma LDL cholesterol, two adult patients being clinically symptomatic.
- Both of these LDL-R gene mutations enrich the spectrum of mutations causing FH in Tunisia. Owing to a high level of inbreeding in the Tunisian population, these findings should provide useful diagnostic tools and allow further studies on genotype/phenotype correlations.

Genetic analysis

Selective polymerase chain reaction (PCR) amplification of the promoter region and of the entire coding sequence of the LDL-R gene (18 exons) was performed on genomic DNA from probands, as previously described. Automated sequencing of purified PCR products (Sephadex G50 spin columns, Boehringer Mannheim, Germany) was performed on both sense and antisense strands using Taq polymerase and fluorescent dinucleotides according to the supplier’s instructions (Applied Biosystems, Perkin Elmer, St Quentin en Yvelines, France). When a mutation was detected, another sequencing reaction

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was performed both on genomic DNA from a relative and from a new PCR product from the proband. Screening relatives for the newly identified mutation was performed after digestion of PCR products with the appropriate enzyme (Appligène, Illkirch, France). The resulting fragments were size separated by electrophoresis on a 2% agarose gel. The Arg3500Gln mutation of \textit{apoB} was not found in any of the patients analysed, using the method described by Hansen et al.\textsuperscript{14}

ApoE E2, E3, and E4 alleles were determined by allele specific oligonucleotide hybridisation (Innogenetics, Gent, Belgium). Common \textit{LPL} mutations (Asp9Asn), (Asn291Ser), and (Ser447Stop) were detected by enzymatic restriction of PCR products as described previously.\textsuperscript{9–11}

LDL-R activity
Analysis of LDL-R activity was performed on cultured skin fibroblasts obtained after skin biopsy from proband II.6 and his mother I.1 from family A, from proband II.1 from family B, and from a healthy donor. Cells were cultured in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 10% fetal calf serum (vol/vol). Induction of the expression of maximal LDL-R activity was obtained by a 48 hour incubation of the cultured fibroblasts in lipoprotein deficient serum. Native LDL used in these studies was isolated from normolipidaemic subjects. Binding, uptake, and degradation of \textsuperscript{125}I-LDL by cultured skin fibroblasts were determined as described elsewhere.\textsuperscript{15} Only results of binding and internalisation at 37°C are shown as data representative of two independent experiments performed in triplicate.

RESULTS
DNA and functional analyses
We analysed two FH probands belonging to two unrelated families A and B living in central and southern Tunisia, respectively. Both probands were born to consanguineous parents (figs 1 and 2).

Proband II.6 from family A was found to be genetically homozygous for a novel missense mutation at codon 127 in exon 4 (fig 1A). A base substitution (TGT to TCT) caused an amino acid change from cysteine to serine, and was designated C127S or FH-Kairouan.

The C127S mutation created a new restriction site for \textit{MnlI} (CCTC), allowing rapid detection of the mutation in all family members. After agarose gel electrophoresis of \textit{MnlI} digested PCR products from exon 4, a 192 bp fragment characterised the normal allele, while a shorter 174 bp fragment characterised the mutant allele (fig 1C, D). This method helped to diagnose unequivocally all carriers of the mutation in family A.

The second proband II.1 from family B was found to be genetically homozygous for a novel missense mutation at codon 245 in exon 5 (fig 2A). A base substitution (GAT to AAT) caused an amino acid change from aspartic acid to asparagine. Genomic

![Figure 1](https://www.jmedgenet.com/family_A.png)

**Figure 1** Family A. (A) Partial LDL-R gene sequence of exon 4 from a control subject (Normal) and from proband II.6 (Mutant). (B) Representative experiment of specific binding and internalisation of \textsuperscript{125}I-LDL at 37°C by fibroblasts from proband II.6, his mother I.1, and a normolipidaemic subject (Normal) (mean of triplicates). (C) Pedigree of family A. (D) Mutation detection by \textit{MnlI} restriction of PCR products from exon 4 in sibs from generation II (lanes 1-7) and a control subject (N).
DNA sequencing in both parents found them to be heterozygous for this mutation. The mutation was designated D245N and named FH-Tozeur. Proband II.1 from family B had less than 2% of normal LDL-R activity in cultured fibroblasts (fig 2B).

**Genotype-phenotype relationship**

In three homozygotes carrying the C127S mutation from family A, levels of plasma LDL cholesterol varied from 746 to 966 mg/dl with a mean of 855 mg/dl (fig 1C). All had extensive xanthomatosis and CAD. They were all carriers of genotype E3E3 for apoE and of wild type for the D9N and N291S variants of the LPL gene. Only proband II.6 was a heterozygous carrier of the Stop447 mutation, suggesting that common modifier alleles were not contributing to plasma lipoprotein levels.

In contrast, plasma LDL cholesterol in proband II.1 from family B, who was homozygous for the D245N mutation, was only 428 mg/dl (fig 2C). Proband B was a carrier of wild type alleles for apoE and for the LPL gene. This woman was free of xanthoma at the age of 20, but she had 40% stenosis of the aortic root. These results suggested dissimilarity in clinical expression between homozygotes carrying different mutations.

Heterozygotes carrying one copy of either of the LDL-R gene mutations (C127S or D245N) had significantly increased plasma LDL cholesterol. Four of them (A1.2, AII.1, AII.5, and AII.7) were heterozygous carriers of the E4 allele. Four C127S carrier heterozygotes (AII.2, AII.1, AII.6, and AII.7) were also carriers of the LPL Stop447 mutation. The mother of proband II.6 (family A) had coronary artery disease (CAD) and high LDL cholesterol level and she was obese (BMI=33.2). The mother of the proband II.1 (family B) had xanthelasma but was free from CAD at the age of 46.

**DISCUSSION**

Two unrelated FH families originating from central and southern Tunisia were found with missense mutations of the LDL-R gene.

The C127S mutation in exon 4, designated FH-Kairouan, changed a highly conserved cysteine in the fourth of the seven tandem cysteine rich repeats involved in the structure of the ligand binding domain. Most missense mutations located in this region have been reported to produce a class 1 (null allele) or a class 2 (transport defective) type of defect. Here, LDL-R activity was abolished in cultured fibroblasts from the proband and was significantly reduced in his mother. All carriers of the mutation had significant hypercholesterolaemia in the family. Therefore, this mutation was found to be responsible for a profound alteration in LDL-R function and an FH phenotype in this family from central Tunisia.

The second mutation, D245N in exon 5, designated FH-Tozeur, changed a highly conserved residue located at the C-terminal end in the sixth of the seven cystein rich repeats that form the binding site for apoB. This mutation, which occurs on a CpG dinucleotide, was previously reported in a Dutch patient, with no mention of any functional consequence in vitro. In addition, a mutation involving the same codon (D245E), FH-Cincinnati-1, was reported in an American homozygous FH patient, which produces a class 2B phenotype. Here less than 2% of the normal LDL-R activity was
found in cultured fibroblasts from the proband. Hypercholesterolemia segregated dominantly with an allelic dosage effect, confirming this mutation as the cause of FH in this family from southern Tunisia.

Genotype-phenotype relationships were analysed in homozygotes and heterozygotes. In three homozygotes carrying the C127S mutation, very high levels of plasma LDL cholesterol were associated with extensive xanthomatosis and CAD. In contrast, plasma LDL cholesterol in the proband who was homozygous for the D245N mutation was nearly two times lower, with no xanthomatosis; however, she had 40% stenosis in the aortic root.

These differences in the LDL cholesterol levels between the two probands support the fact that other genes with lipid lowering effects could interact with the phenotypic expression of the mutation. Recently, Dellaia et al. reported that the mass of LDL cholesterol in Sardinian FH patients who are β-thalassemia carriers was 30% lower than that observed in patients without β-thalassemia trait. Since thalassemia is frequent in central Tunisia, it is quite possible that proband II.1 from family B carries β-thalassemia, which will prompt us to explore this avenue. Common modifier alleles (apoE polymorphism and LPL gene variants) were not found to contribute to any of these homozygous patients.

Heterozygotes carrying one copy of either the C127S or D245N mutation had significantly increased plasma LDL cholesterol. Four of them were heterozygous carriers of the E4 allele, an LDL cholesterol raising allele associated with a higher risk of CAD. However, none of them were affected. Four C127S heterozygotes were also carriers of the LPL Stop 447 mutation. This allele was found to be protective in large human cohorts. The small number of carriers did not allow ruling out of whether these alleles significantly contributed to the clinical expression of FH or not. In addition, obesity could have contributed to the occurrence of CAD in one adult woman from family A. Other genetic or environmental factors could contribute to the phenotypic expression in heterozygotes. Apparent protection of heterozygous FH patients against cardiovascular complications was previously reported in families from the coastal region of Monastir in Tunisia. Mild clinical or biological expression has also been observed in a heterozygous patient from China.29 America,30 and Cuba.31 Recently, a lipid lowering gene was identified on chromosome 13.32 It is not known whether such a cholesterol lowering locus would be involved in these populations or in Tunisia. Another hypothesis could be that the Tunisian Mediterranean diet, low in fat and rich in olive oil, might contribute to the delayed onset of cardiovascular events observed in Tunisian FH heterozygotes living in rural areas. Further investigations are in progress to address this issue.

However, despite protective dietary and life style habits, it has been shown that any person with a defective LDL-R allele has a much greater risk of cardiovascular disease when additive lifestyle or other genetic factors contribute to increase his cardiovascular risk.33 This trend is observed in populations from large cities in Tunisia where a more western lifestyle is adopted (smoking, diet higher in saturated fat and carbohydrates). Therefore, FH heterozygotes may represent a group of high risk subjects frequently found in Tunisia.

In conclusion, the identification of two additional LDL-R mutations in central and southern Tunisia enriches this country specific spectrum of FH causing mutations. They constitute a most useful diagnostic tool and encourage further studies on genotype-phenotype relationships in this specific Mediterranean population.

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Authors’ affiliations
M N Slimane, M Hammani, Laboratoire de Biochimie, Faculté de Médecine, 5019 Monastir, Tunisia
S Lestavel, V Clavey, J C Fruchart, Unité INSERM US545, Institut Pasteur de Lille, Université de Lille 2, 59019 Lille, France
F Maatouk, M H Ben Farhat, Service de Cardiologie, Centre Hospitalier Universitaire, 5019 Monastir, Tunisia
P Benilan, Département de Biologie Moléculaire, Hôpital Saint Antoine, 75571 Paris, France
Correspondence to: Dr M N Slimane, Laboratoire de Biochimie, Faculté de Médecine, 5019 Monastir, Tunisia; Naceur.Slimane@fmm.rnu.tn

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