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

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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.

**MATERIALS AND METHODS**

**Patients**
Proband 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).
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 apoB was not found in any of the patients analysed, using the method described by Hansen et al.

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

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, 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 $^{125}$I-LDL by cultured skin fibroblasts were determined as described elsewhere. 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.

Specific binding and internalisation of LDL at 37°C by fibroblasts of proband II.6 was less than 2% of the activity found in normal cells from a normolipidaemic donor (fig 1B). This proband displayed similar results (data not shown) for degradation at 37°C. As expected, cultured cells from his mother (I.1) showed half of normal LDL-R activity.

The C127S mutation created a new restriction site for MnlI (CCTC), allowing rapid detection of the mutation in all family members. After agarose gel electrophoresis of 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 missense mutation at codon 245 of exon 5 (fig 2A). A base substitution (GAT to AAT) caused an amino acid change from aspartic acid to asparagine. Genomic
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 xanthomatosis 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.1, 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 cysteine-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. Hypercholesterolaemia 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 risk of CAD. However, most of them were not severely 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 smaller 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 heterozygous patients from China,47 America,27 and Cuba.28 Recently, a lipid lowering gene was identified on chromosome 13.29 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.24 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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