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First Report of APOB Gene Variations in Moroccans

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Abstract
The normal clearance of LDL by the LDL receptor is dependent upon normal function of both the LDL receptor and of apoB-100. Accordingly, mutations in the LDL receptor gene and in the apoB-100 gene have been found to cause autosomal dominant hypercholesterolemia. Mutations in the LDL receptor gene cause familial hypercholesterolemia, whereas mutations in the apoB-100 gene cause familial defective apoB-100. In Front lack of data on genotype and phenotype among ADH patients in Moroccan populations, we carried up this study to determine genotype of ADH among subjects of north of Morocco, who have increased rates of TC, LDLc, ApoB and TG. 46 probands were studied. Genomic DNA was isolated us described previously. Direct sequencing is used to determine the mutations on genes LDLR, APOB and PCSK9 and the genotype of the apoE. We have detected for the first time in the Moroccan population three known polymorphisms and V4238A mutation combined with increased TG levels in three probands. This mutation appears to be common polymorphism. Those patients were homozygous of alleles E3/E3. In conclusion, further studies are needed to elucidate the molecular basis of the dyslipidemia observed in the studied probands.

Keywords: Heterozygous familial hypercholesterolemia, genetic diagnosis, lipid phenotype, familial ligand-defective apoB 100, apoE isoform.

Introduction
Autosomal Dominant Hypercholesterolemias (ADHs) (OMIM 143890) are a group of hereditary hypercholesterolemias characterized by elevated levels of low-density-lipoprotein cholesterol (LDLc), tendon xanthomas and increased risk of premature coronary heart disease. The ADHs phenotype results from defects in the LDL receptor gene (LDLR) (familial hypercholesterolemia: FH), and also defects in other genes like apolipoprotein B (apoB) (familial defective apo B: FDB) or proprotein convertase

subtilisin/kexin type 9 (PCSK9) (1,2).

Clinical criteria used to identify patients with ADH include: high plasma levels of total and LDL cholesterol, family history of hypercholesterolemia especially in children, deposition of cholesterol in extravascular tissues such as tendon xanthomas or corneal arcus, and personal and family history of premature CVD (3). Tendon xanthomas are pathognomonic of FH; however, their identification is not always easy and they are considered insensitive diagnostic markers. There are not absolutely predictive clinical criteria for the diagnosis of ADH, and arbitrary criteria must be used. For the USA Make Early Diagnosis to Prevent Early Death (MEDPED) program (4), the diagnostic criteria focus principally on high LDLc levels in the individual, and in family history of hypercholesterolemia with evidence for a dominant transmission (5). The presence of children with hypercholesterolemia increases the diagnostic probability. Recently, the Dutch MEDPED group described a clinical scoring system for the diagnosis of heFH (6). These criteria include personal and familial LDLc levels, history of CVD (coronary, carotid and peripheral arteries), presence of corneal arcus before the age of 45 years and xanthomas. By weighing the occurrence of these clinical signs, alone or in combination with others, a diagnostic scoring table has been constructed in The Netherlands. These criteria seem to be easy to use in clinical practice and consider all the criteria for the diagnosis of ADH. However, even then the diagnosis is not always unequivocal (7).

Few studies have been carried out in Morocco and a small number of mutations for FH were identified and no variation in APOB gene were detected (8-10). No data about ADH mutations in the population from North of Morocco are available so far. Therefore, the purpose of this study was to determine the molecular basis of this disease in this population.

Materials and Methods

Subjects

Forty-six unrelated probands from North-West of Morocco were studied. Diagnosis criteria for ADH probands followed the MEDPED guidelines: high plasma levels of total cholesterol (TC) and low-density lipoprotein cholesterol (LDLc) [TC >250mg/dl and LDLc > 190mg/dl], family history of hypercholesterolemia, presence of xanthomas (tendom, planar, and/or tuberous), and personal and/or family history of premature cardiovascular heart disease (CHD). Secondary causes of hypercholesterolemia, including diabetes, hypothyroidism, nephritic syndrome, etc. were excluded.

Lipid analysis

Serum TC, triglycerides (TG) and high-density lipoprotein cholesterol (HDLc) were assayed by enzymatic methods and apolipoprotein B (apoB) by an immunoturbidimetric method (11). LDLc was calculated using Friedewald formula (12).

Genetic analysis

DNA isolation: Genomic DNA from white blood cells was isolated using a salting-out procedure (13).

To analyze the APOB gene regions where mutations causing FDB have been described. Exons 26 and exon 29, 5 pairs of primers were designed by the software Primer3 (14). PCR amplification was carried out with 50 ng of genomic DNA in a 5 μl reaction volume containing 10X Taq DNA polymerase buffer (HotStarTaq Master Mix Kit), 5x Qsolution (HotStarTaq Master Mix Kit), 50 μmol/l of each dNTP, 0.5 mmol/l of each primer (Sigma) and 1U HotStarTaq DNA polymerase (Qiagen, Hilden, Germany). The thermal cycling conditions were as follows: 95°C for 12 minutes, then 40 cycles of 20 seconds at 95°C, 20 seconds at 62°C, and 40 seconds at 72°C in a PCR apparatus (Applied Biosystems, Veriti™ 96-Well). The sequence reactions were performed using fluorescently labeled dideoxy chain terminations with a Big Dye Terminator ABI Prism kit (Applied Biosystems, Foster City, CA, USA) according manufactory's protocol and analyzed on an Applied Biosystems automated DNA sequencer (model 3730). Sequences were analyzed with the Sequencher package (SeqScan, ABI, USA). The promoter and the 18 exons of the LDLR gene (including about 50 bp of the intronic boundaries) and the PCSK9 gene (promoter and the 12 exons including about 50 bp of the intronic boundaries) were also analyzed in all patients using the same protocol. The genotype of the apoE gene was determined.

Results

Direct sequencing analysis has revealed four LDLR mutations in heterozygosis: one nonsense mutation (C146X) in exon 4 and three missense mutations, one in exon 3 (R57H) and two in exon 17 (V806F and W789L). No mutations were identified in PCSK9 gene. After sequencing APOB gene regions (exons 26 and 29) where mutations causing FDB have been described, four APOB variations were detected: The two silent mutations T3567T and N3579N in the exon 26 and the neutral variation S4211N and the polymorphism V4238A in the exon 29. All variations were detected for the first time in Moroccan population by our group, although it had previously been reported in other populations and are considered not responsible for the disease.

The silent mutation T3567T is a G to A transition at nucleotide 10701 (c.10701G>A) at the exon 26 of APOB gene. It changes codon ACC 3567 to codon ACA code for the same amino acid: the threonine. The mutation was found in heterozygosis in 22 probands. The silent mutation N3579N is a C to T transition at nucleotide 10737 (c.10737C>T) at the exon 26 of APOB gene. It changes codon AAC 3579 to codon AAT code for the same aminoacid: the asparagine. The mutation was found in heterozygosis in 40 probands. The variation S4211N is a G to A transition at nucleotide 13013 (c.13013G>A) at the exon 29 of APOB gene. It changes codon AGC 4211 for serine to codon AAC for asparagine. The variation was found in heterozygosis in 46 probands.

The mutation V4238A is a substitution of T to C at nucleotide 12794 at the exon 29 of APOB gene. This mutation is predicted to result in the production of a 4238 amino acid product, designated V4238A. It changes codon GTA 4238 for valine to codon GCA for alanine (Val4238Ala). It was found in heterozygosis in three probands. This mutation was associated with elevated level of triglycerides in the 3 probands. The three probands were screened for R3500Q mutation in the APOB gene, but none of them had this FDB-causing mutation. No other LDLR gene mutation or PCSK9 gene mutation were identified in those probands. Lipid profile and the Apo E genotype of the three probands are showed in Table 1.

Discussion

The four LDLR mutations in heterozygosis: one nonsense mutation (C146X) in exon 4 and three missense mutations, one in exon 3 (R57H) and two in exon 17 (V806F and W789L) are described in our previous report (15).

Moreover, we have identified, for the first time, in Moroccan population four APOB gene variations considered not responsible for the dyslipidemia. The two silent mutations T3567T and N3579N in the exon 26 and the variation S4211N in the exon 29 were identified in the majority of patients studied with or without cardiovascular disease. Those variations were although described previously in others populations (16-18). They were identified in patients with cardiovascular disease but studies were showed that they not responsible of disease (16-18).

The variation V4238A in the APOB gene is detected for the first time in the Moroccan population. One important result was that the three probands showed increased levels of TG. No one of them shows ischemia complications. In the same way, we analyzed 89 healthy subjects for the presence of the identified mutation. None of them were carrier of the V4238A mutation in the APOB gene. None of three probands had FDB-causing mutation (namely R3500Q mutation in the APOB gene). No other LDLR gene mutation, nor the PCSK9 gene mutation, have been identified in those probands.

The missense mutation V4238A in the APOB gene has been described, in first time, in Norwegian population as mutation causing hypercholesterolemia. The prediction program PolyPhen (19) was employed to assess the pathogenicitie of the missense mutation. These prediction indicated that the mutation do not affect the function of apoB-100. In fact, the authors considered the V4238A mutation as a common polymorphism that not affects the APOB function (20).

Assuming that the V4238A is a common polymorphism, the increased TG level in our patients can be explained by the age. Indeed, probands were 79 old, 70 old and 65 old and previous studies showed that TG levels increase

Table 1. lipid profile and the ApoE genotype of the three probands with *V4238A* variation

Probands	Age (ans)	TC	LDLc	HDLc	TG	apoB	BMI	apoE genotype
1	69	487	300	0.36	228	127	26	E3/E3
2	70	269	200	0.81	225	128	27	E3/E3
3	65	310	250	0.75	219	131	29	E3/E3
Value Limits		<250	<200	>40	<160	135	<30	

TC: Total cholesterol; HDLc: HDL cholesterol; TG: Triglycerides; LDLc: LDL cholesterol; ApoB: Apolipoprotein B; All units are mg/dl, except for BMI (kg/m2).

with age (21). Previous studies are showed that elevated triglyceride levels in some patients with FH have, explained in part by the interaction with other genes as E2/E2 genotype or environmental factors as alcohol, overweight and diabetes mellitus. In our study, Secondary causes of hypercholesterolemia such as diabetes, hypothyroidism, nephritic syndrome, alcoholic patients, were excluded. Consequently, the influence of the environmental factors in phenotype would be eliminating. We, also, determined the apoE genotype, in order to assess its contribution to phenotype. Lipidic profile and the ApoE genotype of the three probands are showed in table 1. The presence of homozygous apoE3 genotype in patients heterozygous of V4238A variation would exclude this gene in their phenotype suppression (22, 23). Indeed, it was shown that the age is positively correlated with the total cholesterol and with the triglycerides and it is the factor has most influence on the lipid parameters (24). This approach cannot exclude the influence of the other genetic factors.

In conclusion, we have identified for the first time in a Moroccan population, three known variations in APOB gene without influence in lipidic profil and variation in APOB gene combined with hypertriglyceridemia but it appears to be a common polymorphism. Further studies are needed to elucidate the molecular basis of the dyslipidemia observed in the studied probands.

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