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Mutational analysis of the LDL receptor and *APOB* genes in Mexican individuals with autosomal dominant hypercholesterolemia

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ABSTRACT

The goal of this project was to identify families with autosomal dominant hypercholesterolemia (ADH) to facilitate early detection and treatment and to provide genetic counselling as well as to approximate the mutational diversity of ADH in Mexico. Mutational analysis of the *LDLR* and *APOB* genes in 62 index cases with a clinical and/or biochemical diagnosis of ADH was performed. Twenty-five mutations (24 *LDLR*, 1 *APOB*) were identified in 38 index cases. A total of 162 individuals with ADH were identified using familial segregation analysis performed in 269 relatives of the index cases. In addition, a novel *PCSK9* mutation, c.1850 C > A (p.Ala617Asp), was detected. The *LDLR* mutations showed the following characteristics: (1) four mutations are novel: c.695 −1G > T, c.1034_1035insA, c.1586 G > A, c.2264_2273del; (2) the most common mutations were c.682 G > A (FH-Mexico), c.1055 G > A (FH-Mexico 2), and c.1090 T > C (FH-Mexico 3); (3) five mutations were identified in 3 or more apparently unrelated probands; (4) three mutations were observed in a true homozygous state; and (5) four index cases were compound heterozygous, and one was a carrier of two mutations in the same allele. These results suggest that, in Mexico, ADH exhibits allelic heterogeneity with 5 relatively common *LDLR* mutations and that mutations in the *APOB* gene are not a common cause of ADH. This knowledge is important for the genotype–phenotype correlation and for optimising both cholesterol lowering therapies and mutational analysis protocols. In addition, these data contribute to the understanding of the molecular basis of ADH in Mexico.

1. Introduction

Cardiovascular disease is the main cause of morbidity and mortality in the world (www.who.int/cardiovascular_diseases/en consulted 2010 November 10), and hypercholesterolemia is associated with a high risk of cardiovascular disease. At least five monogenic types of hypercholesterolemia have been described (for review, 1). Autosomal dominant hypercholesterolemia (ADH) is one of the most frequent Mendelian disorders. ADH is genetically heterogeneous and is mainly due to loss-of-function mutations in the LDL receptor (*LDLR*) gene [2], but loss-of-function mutations in the *APOB* gene or gain-of-function mutations in the *PCSK9* gene are also causes of ADH (for review, 3). In addition, there are two rare forms of autosomal recessive hypercholesterolemia caused by mutations in the *ABCG5/8* (MIM 210250) and *ARH* (MIM 603813) genes (for review, 1).

Mutations in these genes disturb the homeostasis of the total cholesterol and LDL-cholesterol (LDL-C) blood levels. Thereby, in all monogenic types of hypercholesterolemia, the *LDLR* activity in the liver is disrupted, which leads to (a) a decreased rate of LDL removal by the *LDLR*, (b) an increased concentration of total cholesterol and LDL-C, which in turn causes progressive accumulation of LDL in the tendons and arteries and (c) an increased risk of premature atherosclerotic cardiovascular disease [4].

ADH due to mutations of the *LDLR* gene (MIM 606945), named familial hypercholesterolemia (FH, MIM 143890), shows extensive allelic heterogeneity with more than 1000 different mutations described (5,6; www.ac.uk/ldlr/ and www.umd.be/LDLR/). In contrast, only a limited number of mutations have been reported for ADH due to mutations either in the *APOB* gene (MIM 107730), named familial ligand-defective apolipoprotein B-100 (FDB, MIM 144010), or in the *PCSK9* gene (MIM 607786), named HCHOLA 3 (MIM 603776) (for review, 3). Because ADH shows locus heterogeneity, its genetic cause can only be defined using mutational analysis.

ADH is an important public health problem because it is frequent, has severe clinical consequences and reduces life expectancy [7]. There are both therapeutic strategies that aim to decrease the high risk of cardiovascular disease by reducing the LDL-C concentration as well as diverse molecular protocols to define the genetic

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cause of ADH. Programs for identifying and characterising ADH at the molecular level in index cases with ADH followed by familial genetic cascade testing are common in a growing number of countries, primarily in Europe. Advances in the molecular epidemiology of ADH in many countries have been reported [8,9]. In some countries, there is great mutational heterogeneity with many rare pathogenic mutations, whereas in other countries, a few relatively frequent mutations account for an important fraction of the overall prevalence of ADH [8,9].

A project on ADH was recently begun whose principal objectives include (a) defining the genetic cause of ADH in Mexican patients with a clinical and/or biochemical diagnosis and to perform family genetic cascade screening to facilitate early detection, treatment, and genetic counselling, (b) obtaining in the short term, an approximation of the mutational diversity of ADH in Mexico and (c) defining in the long term, the molecular basis of ADH in Mexico. Advances in the project have been presented at several national and one international congress (for review 10,11).

In this paper, the accumulative results of the project are presented.

2. Materials and methods

Screening for the detection of index cases with a clinical and/or biochemical phenotype compatible with a diagnosis of ADH was conducted in 26 hospitals of the Instituto Mexicano del Seguro Social (IMSS) in the metropolitan area of Guadalajara, specifically in 21 and 4 first and second level care hospitals, respectively, and in several departments of the Hospital de Especialidades of the Centro Mèdico Nacional de Occidente [10]. The diagnostic criteria recommended by the International Panel for the Study of Familial Hypercholesterolemia for ADH were applied [7].

Total cholesterol, triglycerides and HDL-C were estimated by standard enzymatic procedures in the central laboratory of the Hospital de Especialidades. LDL-C levels were calculated using an equation. Through several screening modalities and previous exclusion of secondary causes of hypercholesterolemia, a total of 62 index cases, with 59 cases greater than 18 years old and 3 cases less than 18 years old, were identified. To document the vertical transmission of hypercholesterolemia, the lipid values were estimated [10] in almost all the index cases' families.

2.1. DNA analysis

Genomic DNA was extracted from leukocytes of whole blood samples using standard procedures. For the *LDLR* gene, the proximal promoter region, the 18 exons and flanking intron regions were amplified using PCR. A portion of *APOB* exon 26 (nt 10625-10895, **NM_000384**, NCBI Ref Seq) and the exons and flanking intron regions of the *PCSK9* gene (**NG_009061.1**) were also amplified. The PCR products were sequenced in both directions using the Ready Reaction Big Dye Terminator Kit (Applied Biosystems) followed by capillary electrophoresis in an ABI PRISM 310 Genetic Analyzer (Applied Biosystems), and the final analysis was performed with Sequence Analysis Software 5.2 (Applied Biosystems). This protocol was performed in the 62 probands, after informed consent was obtained.

2.2. PCR restriction enzyme analysis (PCR-REA)

Several mutations detected by sequencing analysis were corroborated using PCR-REA. The PCR products, which were incubated with each endonuclease according to the manufacturer's instructions, were subjected to electrophoresis on a 10% acrylamide gel, which was then stained with silver nitrate.

2.3. Family studies

The families of the mutation-positive index cases were analysed only for the specific mutation(s) for each case by sequencing and/or PCR-REA. A total of 269 relatives were analysed. Informed consent was obtained. Cholesterol lowering therapy and genetic counselling were offered to all the hypercholesterolemic individuals.

2.4. In silico predictions

To predict the effect of several mutations on *LDLR* activity, the following web-based tools were used: Poly-Phen (genetics.bwh.harvard.edu/pph/), SIFT (sift.jcvi.org), SNP3D (www.snps3d.org) and Splicing NetGene2 (www.cbs.dtu.dk/services/NetGene2) using the *LDLR* reference NP_000518.1).

2.5. Statistical analysis

Frequencies were calculated for the qualitative variables whereas the mean and standard deviation were estimated for the quantitative variables. To study the differences between the quantitative and qualitative variables, a student t test and chi-squared test, respectively, were used. A p value <0.05 was considered to be significant.

This project was approved by the Comitè Nacional de Investigaciòn en Salud (IMSS).

3. Results

3.1. Clinical and biochemical phenotypes

Screening was performed in 26 hospitals to detect individuals with an ADH phenotype. Sixty-two index cases with a clinical and/or biochemical diagnosis of ADH were detected and studied. Among these individuals, 3 were homozygotes, and 59 were heterozygotes. In the homozygote group, 2 individuals had early onset coronary heart disease; all 3 had xanthomas and arcus cornealis, and 2 had xanthelasma. In the heterozygote group, 17 (29%) individuals had premature coronary heart disease, 18 (30%) had xanthomas based on physical examination, 16 (27%) had arcus cornealis and 9 (15%) had xanthelasma.

All the index cases had LDL-C levels above the 90th percentile for age and gender specific for adult Mexican population [12] when they were detected, except three heterozygotes that had LDL-C levels below the 90th percentile due to they were under cholesterol lowering therapy.

Total cholesterol and LDL-C levels in the homozygote group were higher than those in the heterozygote group ($p < 0.05$) whereas triglycerides and HDL-C levels were lower in the former group ($p < 0.05$). The clinical and biochemical characteristics of the index cases are shown in Table 1.

3.2. DNA analysis

Sequencing analysis was performed to identify ADH-causing mutations in the *LDLR* or *APOB* genes. Mutations were identified in 38 (61%) index cases. The frequency of xanthomas in the mutation-positive group was 45% (17/38), whereas this value was 17% (4/24) in the mutation-negative group ($p < 0.05$). Total cholesterol and LDL-C levels were higher in the mutation-positive heterozygote group than in the mutation negative-group ($p < 0.05$) (Table 1).

Based on sequencing analysis, a total of 25 mutations, 18 missense, 4 small insertion-deletions, 2 nonsense and 1 splicing, were identified. All the mutations were located in the *LDLR* gene except for one missense mutation in the *APOB* gene (Table 2).