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Spectrum of mutations and phenotypic expression in patients with autosomal dominant hypercholesterolemia identified in Italy

Stefano Bertolini [a,**], Livia Pisciotta [a], Claudio Rabacchi [b], Angelo B. Cefalù [c], Davide Noto [c], Tommaso Fasano [d], Alessio Signori [e], Raffaele Fresa [a], Maurizio Averna [c], Sebastiano Calandra [b,*]

[a] Department of Internal Medicine, University of Genova, Viale Benedetto XV, no. 6, I-16132 Genova, Italy
[b] Department of Biomedical, Metabolic and Neural Sciences, University of Modena and Reggio Emilia, Via Campi 287, I-41125 Modena, Italy
[c] Department of Internal Medicine and Medical Specialities, University of Palermo, Italy
[d] Clinical Chemistry, Laboratory and Endocrinology Unit, Azienda Ospedaliera ASMN, IRCCS, Reggio Emilia, Italy
[e] Department of Health Sciences, Section of Biostatistics, University of Genova, Italy

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ABSTRACT

Objective: To determine the spectrum of gene mutations and the genotype–phenotype correlations in patients with Autosomal Dominant Hypercholesterolemia (ADH) identified in Italy.
Methods: The resequencing of *LDLR*, *PCSK9* genes and a selected region of *APOB* gene were conducted in 1018 index subjects clinically heterozygous ADH and in 52 patients clinically homozygous ADH. The analysis was also extended to 1008 family members of mutation positive subjects.
Results: Mutations were detected in 832 individuals: 97.4% with *LDLR* mutations, 2.2% with *APOB* mutations and 0.36% with *PCSK9* mutations. Among the patients with homozygous ADH, 51 were carriers of *LDLR* mutations and one was an *LDLR*/*PCSK9* double heterozygote. We identified 237 *LDLR* mutations (45 not previously reported), 4 *APOB* and 3 *PCSK9* mutations. The phenotypic characterization of 1769 *LDLR* mutation carriers (ADH-1) revealed that in both sexes independent predictors of the presence of tendon xanthomas were age, the quintiles of LDL cholesterol, the presence of coronary heart disease (CHD) and of receptor negative mutations. Independent predictors of CHD were male gender, age, the presence of arterial hypertension, smoking, tendon xanthomas, the scalar increase of LDL cholesterol and the scalar decrease of HDL cholesterol. We identified 13 *LDLR* mutation clusters, which allowed us to compare the phenotypic impact of different mutations. The LDL cholesterol raising potential of these mutations was found to vary over a wide range.
Conclusions: This study confirms the genetic and allelic heterogeneity of ADH and underscores that the variability in phenotypic expression of ADH-1 is greatly affected by the type of *LDLR* mutation.

1. Introduction

The term monogenic hypercholesterolemia is used to indicate a heterogeneous group of Mendelian disorders characterized by the selective increase of plasma low-density lipoprotein cholesterol (LDLc), which causes the accumulation of cholesterol in the arterial wall resulting in accelerated atherosclerosis and premature coronary heart disease (pCHD) [1]. Monogenic hypercholesterolemia includes two genetic subtypes designated Autosomal Dominant Hypercholesterolemia (ADH) and Autosomal Recessive Hypercholesterolemia (ARH), respectively [2].

ADH (OMIM # 143890) is one of the most frequent inherited disorders with an estimated frequency of 1:300/1:500 in most populations [1,2]. Several sets of diagnostic criteria have been adopted for the clinical diagnosis of ADH (MED-PED, Simon Broome Register and Dutch Lipid Clinic Network (DLCN)) [3–5]. ADH is genetically heterogeneous as it can be caused by defects in at least three different genes that encode proteins involved in the hepatic clearance of LDLc mediated by the LDL receptor (LDLR). These defects may be due to mutations in the gene coding for the LDLR (classic Familial Hypercholesterolemia, FH or ADH-1), in the gene coding for the apolipoprotein B (Familial Defective apoB, FDB or ADH-2) and the gene coding for the enzyme PCSK9 (ADH-3) [1–3,6]. Mutations in *LDLR* gene are the most frequent cause of ADH

* Corresponding author. Tel.: +39 (0)59 2055423; fax: +39 (0)59 2055426.
** Corresponding author. Tel.: +39 (0)10 3537992; fax: +39 (0)10 3537797.
E-mail addresses: stefbert@unige.it (S. Bertolini), sebcal@unimore.it (S. Calandra).

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(from 90 to 95%); mutations in *APOB* account for 3–6% and mutations in *PCSK9* are found in less than 1% of patients [7,8]. In approximately 15–19% of patients with the clinical diagnosis of definite ADH no mutations in the three candidate genes have been detected, suggesting the presence of other yet unknown genes [6,7].

A large number of mutations in *LDLR* gene have been reported in patients with ADH-1 (online FH data base www.ucl.ac.uk/ldlr/LOVD.1.10) [9,10]. With the exception of some populations where few mutations account for most ADH-1 patients (founder effect), in most populations there is a great heterogeneity of *LDLR* mutations [9,11]. By contrast only few mutations of *APOB* and *PCSK9* were found to be responsible for ADH-2 and ADH-3, respectively [6,12].

The aims of this study were the assessment of the molecular bases of ADH in a cohort of patients attending the Italian Lipid Clinics at the University Hospitals of Genova, Modena and Palermo and the systematic analysis of the clinical features of mutation positive ADH patients.

2. Methods

2.1. Subjects

This study includes 1018 unrelated index patients (478 males and 540 females, 43.9 ± 17.3 years of age, range 2–86 years) with the clinical diagnosis of heterozygous ADH, who, over the last two decades, had been investigated at the Lipid Clinics of the University Hospitals of Genova, Modena and Palermo. The clinical diagnosis of ADH was made by adopting a combination of criteria, including: i) untreated plasma low-density lipoprotein cholesterol (LDLc) level above the 95th percentile of the distribution in the population (stratified for gender and age) [13,14] and plasma triglyceride levels below 2.8 mmol/L, after the exclusion of secondary hypercholesterolemias; ii) the presence of tendon xanthomas in the index patient or in at least one family member or the presence of hypercholesterolemia in some prepuberal children of the family; iii) premature coronary heart disease (pCHD), before 55 years of age in males and 65 in females, in the index subject or in one first-degree relative; iv) vertical transmission and bimodal distribution of hypercholesterolemia in the family. The criteria specified in ii) and iii) were fulfilled only in approximately 45% of the index patients. In the remaining 55% of index cases these criteria were not fulfilled for the absence of tendon xanthomas, the lack of reliable family data or the absence of pCHD in the family.

In order to obtain a probability score of having ADH we retrospectively classified the patients according to the clinical criteria of the Dutch Lipid Clinic Network (DLCN) [15]. According to DLCN score, 473 subjects (46.5%) were classified as “definite ADH”, 257 (25.2%) as “probable ADH” and 288 (28.3%) as “possible ADH”, respectively. Data on current and previous smoking (SMO), arterial hypertension (AH), diabetes or other diseases with impact on cardiovascular risk were recorded. The few patients with diabetes (6 patients with type II diabetes) and those carrying the β-thalassemia trait [16], were excluded from the clinical survey as both conditions are known to affect plasma lipoprotein levels.

In addition 50 patients were referred to the Lipid Clinics with the clinical diagnosis of probable homozygous ADH (HO-ADH) on the basis of the following criteria: i) plasma LDLc ≥13 mmol/L; ii) the presence of tendon and cutaneous xanthomas in infancy and iii) history of hypercholesterolemia in both parents. In addition, two children were assumed to have homozygous ADH in view of the presence of extensive cutaneous xanthomatosis, despite a LDLc level (10.21 and 12.88 mmol/L, respectively) below the cut-off of 13 mmol/L.

All subjects found to have molecularly defined ADH underwent cardiovascular examination, including exercise ECG, thallium test or stress echocardiography and ultrasound evaluation of the carotid arteries. In some cases coronary angiography was also performed. The subjects were considered positive for CHD (CHD+) as reported previously [17] and described in the supplemental methods.

Informed written consent was obtained from the index subjects and their family members or, in the case of children, from their parents. The study protocol was approved by the institutional human investigation committee of each participating institution.

2.2. Biochemical analyses

Plasma concentrations of total cholesterol (Tc), triglycerides (Tg) and high-density lipoprotein cholesterol (HDLc) were measured by standard methods [18]. LDLc was calculated by the Friedewald’s formula.

2.3. LDL receptor activity

The assay of LDL receptor activity in cultured skin fibroblasts was performed as previously reported [19]. This assay was performed in all patients found to carry two *LDLR* mutant alleles and in a few other patients either simple heterozygous for *LDLR* mutations or double heterozygous for *LDLR* and *PCSK9* mutations [19,20].

2.4. Sequence analysis of candidate genes for ADH

Genomic DNA was extracted from peripheral blood by a standard procedure. *LDLR* gene was analyzed by direct re-sequencing [20]. The search for major gene rearrangements was performed by Southern blot analysis [19] or by multiple ligation-dependent probe amplification (MLPA) (MRC Holland, Amsterdam, The Netherlands) [21]. The re-sequencing of the whole *PCSK9* gene and of the 3′ end of exon 26 of *APOB* gene (from c.9216 to c.11788 +152 nt of intron 26) were performed in: i) all patients negative for *LDLR* mutations; ii) in all patients with the clinical features consistent with the diagnosis of homozygous ADH in whom only one *LDLR* mutant allele had been found [20].

The mutations were designated according to the Human Genome Variation Society, 2012 version (http://www.hgvs.org/mutnomen/recs-DNA.html). LDLR, APOB and PCSK9 protein sequence variants were designated according to http://www.hgvs.org/mutnomen/recs-prot.html.

2.5. Northern blot analysis and reverse transcription-PCR amplification (RT-PCR)

The analysis of LDLR mRNA, was performed in ADH-1 subjects carrying major gene rearrangements or intronic mutations suspected to affect splicing, whose skin fibroblasts were available in our cell bank [19].

2.6. In silico analysis

The *in silico* prediction of the effect of the missense mutations of *LDLR*, *APOB* and *PCSK9* genes was performed using PolyPhen-2 (http://genetics.bwh.harvard.edu/pph2/), SIFT Human Protein (http://sift.jcvi.org/), refined SIFT [9] and Mutation Taster (http://neurocore.charite.de/MutationTaster/).

The *in silico* prediction of the effect of intronic variants was performed using NetGene2 (http://www.cbs.dtu.dk/services/NetGene2), Human Splicing Finder (http://www.umd.be/HSF/HSF.html) and Automated Splice Site Analysis (https://splice.uwo.ca).