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Update of the Portuguese Familial Hypercholesterolaemia Study

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ABSTRACT

The main aim of the Portuguese Familial Hypercholesterolaemia Study is to identify the genetic cause of hypercholesterolaemia in individuals with a clinical diagnosis of Familial Hypercholesterolaemia (FH). A total of 1340 blood samples were collected from 482 index patients and 858 relatives with the collaboration of clinicians from several hospitals all over the country. The genetic diagnosis of FH in this study is based on the analyses of three genes: *LDLR*, *APOB* and *PCSK9*. In the last 10 years, the Portuguese FH Study identified a genetic defect in a total of 171 index patients, corresponding to an overall of 48% of the total received cases with clinical diagnosis of FH. Although the Simon Broome FH register criteria have been adapted to our study, 59 patients that did not fulfil all criteria were included in the study and a mutation causing disease was identified in 8 of these patients. In the LDLR gene were found 80 different mutations in 165 unrelated index patients: 159 heterozygous, 3 compounds heterozygous and 3 true homozygous. The APOB p.Arg3527Gln and the PCSK9 p.Asp374His mutation were not found in any of our patients since our last report, but a novel mutation in the APOB gene, predicted to cause a single amino acid substitution p.Tyr3560Cys, was found in one patient. The cascade screening in relatives of these 171 index patients allowed the identification and genetic characterization of a total of 404 FH patients in Portugal.

1. Introduction

Familial Hypercholesterolaemia (FH) is an autossomal dominant disorder clinically characterized by high levels of LDL-associated cholesterol in plasma. This accumulation causes its deposition on arteries and tendons leading to accelerated atherosclerosis and increased risk of premature coronary heart disease (CHD) [1].

FH usually results from mutations in three different genes involved in lipid metabolism [2]. These include, most commonly, mutations in the low density lipoprotein receptor gene (LDLR), less commonly in the apolipoprotein B (APOB) gene and rarely in the proprotein convertase subtilisin/kesin type 9 (PCSK9) gene [3]. At present, more than 1000 mutations have been described worldwide in the LDLR gene (http://www.ucl.ac.uk/fh) and the residual LDL receptor activity varies considerably between those [4]. Mutations in exons 26 and 29 of the APOB gene have been identified in FH patients, the most common of which is the nucleotide change c.10708G>A, predicted to lead to the amino acid substitution p.Arg3527Gln [5]. From the PCSK9 mutations reported worldwide [6] none was found in the Portuguese population except the mutation, p.Asp374His, located at the same codon as the p.Asp374Tyr described previously [6], but leading to a different amino acid substitution.

In 1999, it was first established by the Portuguese FH Study, a DNA diagnosis for patients with clinical diagnosis of FH and cascade screening in relatives of the affected index patients. This study has been based at the National Institute of Health, Lisbon, where the biochemical characterization and DNA diagnosis have been performed with no costs for the patient/medical institution partly due to a collaboration established in 2006 with the Portuguese Society of Cardiology. The number of index patients has expanded significantly since then due an increasing network of 24 clinicians located throughout several hospitals in Portugal. The genetic diagnosis of FH is performed in three phases and an optional fourth phase. Phase I comprises screening for the common mutations in APOB gene and analysis of LDLR gene. Phase II includes identification of large rearrangements in the LDLR gene using MLPA technique. Phase III consists in screening of PCSK9 gene and is only performed if no mutation was found in phases I and II. Phase IV is only performed when putative splicing mutations, not described before or without functional studies, are found. RNA from peripheral blood mononuclear cells (PBMC) is extracted and the cDNA is analysed to identify the effect of the alteration on the splicing mechanism.

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Table 1
New LDLR mutations identified in the Portuguese FH patients since last mutation report in 2008.

Location	Nucleotide change	Protein	Predicted effect	References
Promoter	c.−135C>G		LDLR not produced	[9]
Promoter	**c.−13A>G**		**LDLR not produced**	**Novel**
Exon 2	**[EX2_3del]**		**Large deletion of 2 exons**	Novel[a]
Intron 2	**c.190+4insTG**		**Splicing error**	**Novel**
Exon 3	c.274C>G	p.Gln92Glu		[10]
Exon 4	c.427T>C	p.Cys143Arg		[11]
Exon 4	**c.473C>G**	**p.Ser158Cys**		**Novel**
Exon 4	c.502G>C	p.Asp168His		[12]
Exon 4	c.551G>A	p.Cys184Tyr		[13]
Exon 4	c.590G>T	p.Cys197Phe		[14]
Exon 8	c.1176C>A	p.Cys392X	Stop codon	[15]
Exon 9	c.1322T>C	p.Ile441Thr		[16]
Intron 9	c.1359−5C>G		Splicing error	[9]
Exon 10	c.1325A>G	p.Tyr442Cys		[17]
Exon 10	**c.1403T>A**	**p.Val468Asp**		**Novel**
Exon 11	c.1599G>A	p.Trp533X	Stop codon	[18]
Exon 12	**c.1756delT**	**p.Ser586GlnfsX78**	**Truncated peptide**	**Novel**
Intron 12	c.1846−1G>A		Splicing error	[12]
Exon 13	c.1876G>A	p.Glu626Lys		[19]
Exon 13	**c.1936delC**	**p.Leu646TyrfsX18**	**Truncated peptide**	**Novel**
Exon 13	c.1966C>A	p.His656Asn		[20]
Exon 14	c.2054C>T	p.Pro685Leu		[21]
Intron 14	c.2140+1G>A		Splicing error	[22]
Exon 15	**c.2146G>A**	**p.Glu716Lys**		**Novel**
Exon 16	**[EX16_18del]**		**Large deletion of 3 exons**	Novel[a]
Exon 16	**c. 2385delC**	**p.Pro795ProX20**	**Truncated peptide**	**Novel**
Exon 16	c.2389G>T	p.Val797Leu	Splicing error	[9]

Mutations not described before are in bold.
[a] Similar large rearrangements were already described but since the deletions breakpoints are unknown it is possible that the Portuguese rearrangements are different from the previously described.

2. Methods

2.1. Patients

A total of 1340 blood samples were collected from 482 index patients and 858 affected and unaffected relatives. The 482 index patients (318 adults and 164 children) were referred to our lab following the Simon Broome criteria for FH, as presented before [7,8].

2.2. Biochemical characterization

The biochemical parameters, including total cholesterol, LDL-cholesterol, HDL cholesterol, triglycerides, apolipoprotein AI, apolipoprotein B and lipoprotein (a), were determined for all individuals in an Hitachi 911 (Boehringer, Mannheim, Roche) until 2007 and in Cobas Integra 400 plus (Roche) since then, by an enzymatic colorimetric method.

2.3. DNA isolation

Genomic DNA was isolated from whole blood EDTA samples, using the Wizard® Genomic DNA Purification kit (Promega) according to the manufacturer's instruction.

2.4. DNA analysis

The promoter region and the 18 exons of the LDLR gene were amplified from genomic DNA by polymerase chain reaction (PCR) and screened for sequence alterations using denaturing high pressure liquid chromatography (DHPLC) and automated sequencing. Large rearrangements in the LDLR gene were identified by multiplex ligation-dependent probe amplification (MLPA) technique using the SALSA® MLPA® kit P062 LDLR (MRC-Holland). Screening for the common mutations in the APOB gene was performed by PCR amplification and direct sequencing of exons 26 and 29. The 12 exons of PCSK9 gene were amplified by PCR and analysed by direct sequencing. All the described techniques were performed as reported before [8].

2.5. RNA extraction and reverse transcriptase (RT) reaction

PBMC were isolated from 8 ml of fresh blood collected in CPT tube (cell preparation tube with sodium citrate, BD Vacutainer®) by centrifugation for 30 min at 2800 rpm, 4 °C (separation of PBMC must occur during the first 2 h after collection). The upper layer containing plasma and PBMC was transferred to a 15 ml falcon tube. The PBMC were obtained by centrifugation for 10 min at 1400 rpm, 4 °C. The mononuclear cells (pellet) were then washed with PBS by centrifugation as above. Total RNA was extracted with the RNeasy® Mini Kit (Qiagen), including the optional DNase I incubation as described in the kit protocol. This procedure yielded 6.5–20 μg of total RNA (200–600 ng/μl). To prepare cDNA, 1 μg of RNA was reverse transcribed with TaqMan Reverse Transcription (Applied Biosystems) as described in the kit protocol [9].

2.6. RT-PCR and analysis of PCR fragments

Amplification of the cDNA fragments of interest was performed in a T3000 thermocycler (Biometra) as reported before [9].

3. Results

3.1. Clinical analysis

Biochemical data referring to total cholesterol and LDL-cholesterol, for the FH patients genetically identified in Portugal (before treatment values were only available for 351 Portuguese FH patients) is shown in Supplementary Fig. S1A.

Coronary heart disease is present in 49 of the total Portuguese FH patients genetically identified (17% of the adult FH patients). Premature CHD analysis of the 49 FH patients distributed according