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Molecular studies in Portuguese patients with Smith–Lemli–Opitz syndrome and report of three new mutations in *DHCR7*

M.L. Cardoso [a,*], A. Balreira [a], E. Martins [b], L. Nunes [c], A. Cabral [d], M. Marques [c],
M. Reis Lima [a], J.S. Marques [e], A. Medeira [d], I. Cordeiro [d], S. Pedro [f], M.C. Mota [b],
C. Dionisi-Vici [g], F.M. Santorelli [g], C. Jakobs [h], P.T. Clayton [i], L. Vilarinho [a]

[a] *Instituto de Genética Médica Jacinto de Magalhães, Oporto, Portugal*
[b] *Hospital Maria Pia, Oporto, Portugal*
[c] *Hospital D. Estefânia, Lisboa, Portugal*
[d] *Hospital Santa Maria, Lisboa, Portugal*
[e] *Centro Hospitalar de Gaia, Vila Nova de Gaia, Portugal*
[f] *Instituto Nacional de Saúde Ricardo Jorge, Lisboa, Portugal*
[g] *Bambino Gesù Hospital, Rome, Italy*
[h] *Metabolic Unit, Department of Clinical Chemistry, VU University Medical Centre, Amsterdam, The Netherlands*
[i] *Institute of Child Health, University College London, England, UK*

Abstract

Smith–Lemli–Opitz syndrome (SLO) is an autosomal recessive disorder characterised by craniofacial dysmorphism, mental retardation, multiple congenital anomalies, and increased levels of 7-dehydrocholesterol (7-DHC) in body tissues and fluids. SLO is caused by mutations in the *DHCR7* gene which encodes 7-dehydrocholesterol reductase, the last enzyme of cholesterol biosynthesis pathway. In our investigation, we screened 682 dysmorphic/mentally retarded Portuguese patients for abnormal levels of 7-DHC in blood by UV spectrometry. We identified six unrelated patients with SLO (0.87% of total). Mutational analysis of the *DHCR7* gene led to the identification of seven distinct mutations, three of which are new (F174S, H301R, and Q98X). The common IVS8-1G > C and T93M variants together with the H301R accounted for 70% of the all SLO alleles in our population. Our findings contribute to the variegate array of pathological changes in the *DHCR7* gene among different European populations.
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Introduction

Smith–Lemli–Opitz syndrome (SLO), OMIM #270400, is one of the six known disorders associated with altered post-squalene cholesterol biosynthesis. SLO is an autosomal recessive disorder caused by a deficiency of 7-dehydrocholesterol reductase (3β-hydroxysteroid-Δ^7-redutase, EC. 1.3.1.21) which catalyses the last step of Kandutsch–Russel pathway, that is the conversion of 7-dehydrocholesterol (7-DHC) to cholesterol [1,2]. Although this metabolic disease was first described in 1964 [3] as a syndrome of mental retardation and multiple malformations, it took about 30 years to identify the underlying enzymatic defect [2]. Surveys of large series of patients showed a constellation of severe birth defects affecting most organ

systems [1] including microcephaly, dysmorphic facies, limb abnormalities, cryptorchidism, mental retardation, and poor growth (see also [4] for details on the clinical spectrum).

The remarkable variability in clinical expression does not allow a definite diagnosis in many cases [5], however, a clinical suggestion of SLO is usually confirmed by demonstrating a marked increase of 7-DHC in plasma or tissue [2,6] by employing ultraviolet spectrometry [6–8], gas chromatography with flame ionisation detection (GC-FID), tandem-mass spectrometry [9], gas chromatography–mass spectrometry (GC–MS) [2,10–12] or time-of-flight secondary ion mass spectrometry [13]. The rise in 7-DHC is generally considered the biochemical hallmark of SLO.

The *DHCR7* gene, encoding 3β-hydroxysteroid-Δ^7-reductase, is located on chromosome 11, spans about 14 kb of genomic sequence, and contains nine exons and eight introns [14]. The first two exons are noncoding and the starting codon is located in exon 3. Exon 9 is the largest exon and contains the stop codon and the two polyadenylation sites [15]. So far, over 90 different mutations have been described in SLO patients [16].

The aims of this study were to identify the spectrum of mutations in Portuguese patients with SLO and to develop a rapid molecular genetic test by using denaturing high-performance liquid chromatography (DHPLC) analysis of the *DHCR7* gene.

Materials and methods

Samples

Over a period of 3 years, we have received 682 requests to look for elevated 7-DHC. Median age of patients who underwent a biochemical screening was 3 years (range 1 day–16 years). Usually the accompanying clinical data were poor. Recurrent clinical features in our cohort were as follows: microcephaly (33% of cases), mental retardation (28%), dysmorphic features (14%), malformation syndrome (5.3%), 2/3 syndactyly of toes (2.6%), cleft palate (1.2%), hypospadias/ambiguous genitalia in males (0.9%), low levels of serum cholesterol (0.3%), suspicion of metabolic disorder (0.9%), and combinations of the previously features (13.8%). In two cases, a clinical and biochemical diagnosis of SLO had already been demonstrated [17]; these children were included as positive controls for mutation analysis.

Blood samples were collected into tubes with EDTA/heparine. After centrifugation plasma was stored at −20 °C until analysed. When available, blood cells were kept at 4 °C and later used for DNA extraction of positive cases (QIAamp DNA Blood Mini Kit, Qiagen 51104).

UV spectrometric screening for abnormal levels of 7-DHC

The biochemical screening for SLO was adapted from the method proposed by Honda et al. [8]. An aliquot of plasma or serum was deproteinised with equal volume of ethanol and the neutral lipids extracted by vortexing with spectral quality *n*-hexane. After centrifugation, the organic phase was analysed in a Shimazu 160A spectrophotomer and the spectrum of the organic extract between 220 and 320 nm, was registered. One standard solution of 7-DHC was tested at the same time showing the characteristic 4 absorption maxima at 260, 271, 282, and 294 nm [6] and this spectrum was compared with those of the tested samples.

GC–MS analysis of sterols

Plasma/serum samples were analysed by GC–MS for quantification of cholesterol, 7-DHC, and 8-dehydrocholesterol (8-DHC) when the UV screening test was positive. GC–MS was also performed in patients whose clinical presentation made a deficiency in the cholesterol biosynthesis pathway highly suspicious. The GC–MS method was adapted from Kelley [10]. After saponification with KOH, neutral sterols from plasma were extracted with *n*-hexane by vortexing. The organic layer was removed, dried under nitrogen, and derivatised with *bis*-trimethylsilyl-trifluoroacetamide (BSTFA) and pyridine. One microlitre of the derivative mixture was then analysed by GC–MS in a Hewlett–Packard 6890/5973 series in two modes: full-scan (for further confirmation of the identity of the compounds based on their mass-spectra) and in selective-ion-mode (SIM) for more accurate quantification of cholesterol 7-DHC and 8-DHC.

Mutation analysis

Mutation analysis of *DHCR7* was performed in patients who manifested elevated levels of 7-DHC and in the two previously identified SLO cases (positive controls) [17]. When possible, DNA from parents was also sequenced to show segregation of the mutation and to reconstruct the whole haplotype. A panel of at least 100 ethnically matched control chromosomes was screened to substantiate pathogenicity of new mutations.

Genomic DNA was extracted from peripheral blood and used for PCR. Exons 3–9 and their intron–exon boundaries were amplified with Ready-To-Go PCR Beads (Amersham Biotech, Uppsala, Sweden) according to the manufacturer's indications using primers outlined in Table 1.

PCR products were gel purified from 2% agarose gel using Quantum Prep Freeze'N Squeeze DNA Gel Extraction columns (Bio-Rad, USA) and sequenced using the ABI Genetic Analyser 310 with Big Dye Terminator Cycle v1.1 sequencing Ready reaction kit