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ARTICLE

The desmosterolosis phenotype: spasticity, microcephaly and micrognathia with agenesis of corpus callosum and loss of white matter

Jenny Zolotushko[1,6], Hagit Flusser[2,6], Barak Markus[1], Ilan Shelef[3], Yshaia Langer[1], Maura Heverin[4], Ingemar Björkhem[4], Sara Sivan[1] and Ohad S Birk*[,1,5]

Desmosterolosis is a rare autosomal recessive disorder of elevated levels of the cholesterol precursor desmosterol in plasma, tissue and cultured cells. With only two sporadic cases described to date with two very different phenotypes, the clinical entity arising from mutations in 24-dehydrocholesterol reductase (*DHCR24*) has yet to be defined. We now describe consanguineous Bedouin kindred with four surviving affected individuals, all presenting with severe failure to thrive, psychomotor retardation, microcephaly, micrognathia and spasticity with variable degree of hand contractures. Convulsions near birth, nystagmus and strabismus were found in most. Brain MRI demonstrated significant reduction in white matter and near agenesis of corpus callosum in all. Genome-wide linkage analysis and fine mapping defined a 6.75 cM disease-associated locus in chromosome 1 (maximum multipoint LOD score of six), and sequencing of candidate genes within this locus identified in the affected individuals a homozygous missense mutation in *DHCR24* leading to dramatically augmented plasma desmosterol levels. We thus establish a clear consistent phenotype of desmosterolosis (MIM 602398).
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INTRODUCTION

Aberrations of the final stages of cholesterol biosynthesis can lead to either Smith–Lemli–Opitz syndrome (SLOS) or to desmosterolosis, depending on the enzymatic defect in the pathway. Although the clinical entity of SLOS is well defined, that of desmosterolosis is yet unresolved. Human desmosterolosis (MIM 602398) is an autosomal recessive disorder of elevated levels of the cholesterol precursor desmosterol in plasma, tissue and cultured cells, stemming from mutations in 24-dehydrocholesterol reductase (*DHCR24*). To date, only two clinical cases of this biochemical disorder have been described. Although *DHCR24* mutations of both alleles were demonstrated in both cases,[1] the clinical phenotypes of the two cases are very different. Through linkage analysis and biochemical studies of four individuals of a consanguineous Bedouin Israeli kindred, we now demonstrate a consistent phenotype of this rare human disorder.

MATERIALS AND METHODS

Patient's samples

Consanguineous Israeli Bedouin kindred (Figure 1) was clinically and genetically investigated. Clinical data of two deceased individuals (IV8, V10) was not available. The four surviving affected individuals of the kindred and 14 of their first-degree relatives (parents and siblings) were included in the study. The study was approved by the Institutional Review Board of Soroka Medical Center and informed consent was obtained from all participants or their legal guardians.

Clinical evaluation

The medical records of all surviving four affected individuals were reviewed, and all had undergone careful clinical evaluation by a pediatric neurologist and a clinical geneticist followed by thorough biochemical laboratory testing and MRI.

Ruling out of homozygosity in loci of known genes

Homozygosity of affected individuals at loci of the genes known to be associated with inherited defects of white matter or agenesis of corpus callosum was tested using microsatellite markers as previously described.[2] Microsatellite markers were derived from Marsheld maps. Intronic primer pairs were designed with the Primer3 (version 0.4.0) software (http://fokker.wi.mit.edu/primer3/), based on DNA sequences obtained from UCSC Genome Browser (sequences available on request). PCR products were separated on polyacrylamide gel using silver staining for detection.

Linkage analysis

Genome-wide scan was carried out using GeneChip Human Mapping 500K Array Set, Nsp Array containing 250 000 SNPs (Affymetrix, Fremont, CA, USA) according to the Affymetrix GeneChip Mapping Assay protocol as previously described.[3] Genomic DNA (250 ng) from each subject was processed and labeled with reagents and protocols supplied by the manufacturer. Homozygosity by descent analysis was carried out using an in house tool for homozygosity mapping (Marcus *et al*, in preparation). Only one significant region on 1p33-1p32.3 was detected. Fine mapping of a locus on 1p33-1p32.3, haplotype analysis and elimination of candidate genes were performed by

[1]The Morris Kahn Laboratory of Human Genetics at the National Institute of Biotechnology in the Negev, Ben-Gurion University of the Negev, Beer Sheva, Israel; [2]Division of Pediatrics at Soroka Medical Center, Ben-Gurion University of the Negev, Beer Sheva, Israel; [3]Diagnostic Imaging Institute at Soroka Medical Center, Ben-Gurion University of the Negev, Beer Sheva, Israel; [4]Division of Clinical Chemistry, Department of Laboratory Medicine, Karolinska University Hospital, Huddinge, Karolinska Institutet, Stockholm, Sweden; [5]Genetics Institute at Soroka Medical Center, Ben-Gurion University of the Negev, Soroka University Medical Center, Beer Sheva, Israel
*Correspondence: Professor OS Birk, Genetics Institute at Soroka Medical Center, Ben-Gurion University of the Negev, Soroka University Medical Center, POB 151, Beer Sheva 84101, Israel. Tel: +97 28 640 3439; Fax: +97 28 640 0042; E-mail: obirk@bgu.ac.il
[6]These authors contributed equally to this study.
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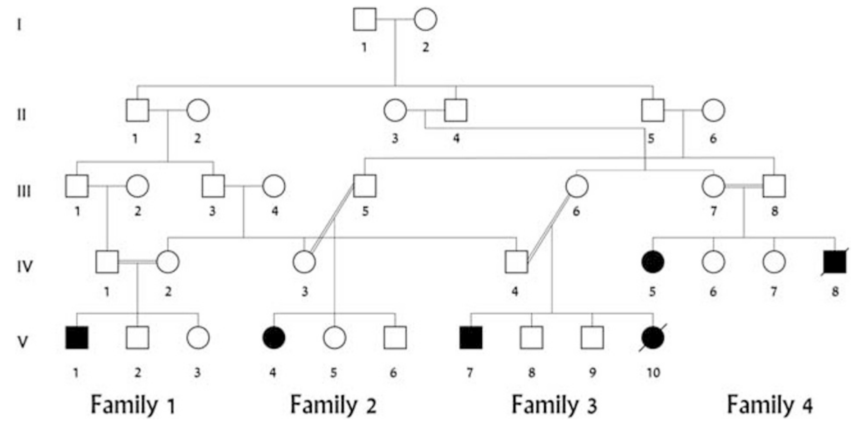

Figure 1 Pedigree of the family studied. The four surviving affected individuals were available for genetic analysis. The autosomal recessive pattern of inheritance because of a likely founder effect can be observed.

genotyping of additional microsatellite markers derived from Marsheld maps or novel markers designed based on Tandem Repeats Finder program and the UCSC Human genome database, in all affected individuals included in the study and their close relatives. Multipoint LOD score calculation using SUPERLINK[4] (http://bioinfo.cs.technion.ac.il/superlink-online/) was carried out for markers *D1S2720*, *D1S2134*, *D1S2824*, *D1S1616*, *D1S2748*, *D1S197*, *D1S427*, *Ch.1_51819Kb*, *D1S231*, *Ch.1_53080Kb*, *Ch.1_54036*, *Ch.1_54982*, *D1S200* and *D1S2742* on 1p33-1p32.3 (markers *Ch.153080Kb*, *Ch.154036Kb* and *Ch.154982Kb* were designed using tandem repeats in UCSC genome browser. Sequences are available on request.). The calculations were carried out assuming an autosomal-recessive mode of inheritance with penetrance of 0.99, a disease mutant gene frequency of 0.01 and a uniform distribution of allele frequencies.

Sequence analysis

Genomic DNA of all participants was extracted from peripheral lymphocytes using standard methods.[3] EBV transformation of lymphocytes of affected individuals was carried out as previously described.[5] RNA was extracted from cultured cells of EBV-transformed lymphoblastoid cell lines using the RNeasy Mini Kit (Qiagen, Petach Tikva, Israel) and cDNA was reverse transcribed by the Verso RT-PCR Kits (TAMAR, Mevaseret Zion, Israel) according to the protocol of the manufacturer.[6] Primer pairs for cDNA and/or exons of genomic DNA (including flanking intron sequences) of eight genes in the putative 1p33-1p32.3 locus were designed based on the known mRNA and genomic sequences using Primer3. Primer sequences and PCR conditions are available on request. PCR products were directly sequenced using ABI PRISM 3730 DNA Analyser according to the protocols of the manufacturer (Applied Biosystems, Foster City, CA, USA). Sequence variations were confirmed by bidirectional sequencing.

Mutation detection-restriction analysis

Testing for the *DHCR24* mutation in the entire family and controls was carried out using restriction analysis, based on the fact that the mutation abrogates an AflIII restriction site. PCR amplification of genomic DNA using this primer set gave a 161 bp fragment, generating AflIII (NEB) differential cleavage products of the mutant (uncut, 161 bp) *versus* wild-type alleles (100 and 61 bp). Fragments were separated by electrophoresis on 3% agarose gel. PCR amplification primers: 5′-CTCACCCTCTGTCCTGTGGT-3′ and 5′-CCAGAATGTC CATCAGGTTG-3′.

Functional and biochemical assays

Cholesterol and desmosterol concentrations were determined in plasma collected from four family members: two fathers (obligatory healthy carriers of the *DHCR24* mutation) and their affected sons. Plasma (10 μl) was processed together with 2 μg of deuterium labeled cholesterol as an internal standard.[7] Following hydrolysis, lipid extraction and derivation, the samples were analyzed by gas chromatography mass spectrometry using the selected ion monitoring mode. The following ions were monitored: *m/z* 464 (deuterium labeled cholesterol), *m/z* 458 (cholesterol) and *m/z* 343 (desmosterol). Plasma from one of the affected sons was also processed for oxysterol analysis. This sample was run in scan mode to search for the presence of 27-hydroxydesmosterol.

Table 1 Clinical characteristics of the affected individuals

Patient number	*V1*	*V4*	*V7*	*IV5*	*Andersson et al*[17]
Gender	*Male*	*Female*	*Male*	*Female*	*Male*
Present age (years)	6	11	4	4	3 (at publication)
Microcephaly	+	+	+	+	+
Microretrognathia	+	+	+	+	+
Psychomotor retardation	+	+	+	+	+
Spasticity with contractures of hands	+	+	+	+	+
Partial/full agenesis of corpus callosum	+	+	+	+	+
Ventriculomegaly with thinning of white matter	+	+	+	+	
Nystagmus and strabismus	+		+	+	
Convulsions	+	+	+		
Failure to thrive	+	+	+	+	+

RESULTS

Clinical evaluation

A consistent severe autosomal recessive neurological phenotype was identified in four individuals of large consanguineous Israeli Bedouin kindred (Figure 1). Four of the six affected individuals (Figure 1, individuals V1, V4, V7 and IV5) were alive and available for thorough clinical investigation. Failure to thrive, psychomotor retardation, microcephaly, micro-retrognathia and spasticity with variable degree of contractures of hands were seen in all patients, whereas severe convulsions near birth, as well as nystagmus and strabismus were evident in most. Brain MRI of all surviving affected individuals demonstrated significant reduction in white matter and partial or complete agenesis of corpus callosum (Table 1). Representative MRI images of two cases (V7, IV5) at age of 1.5 and 2 years are shown in Figure 2, demonstrating microcephaly (Figure 2a), a thin corpus callosum (Figures 2a and b), bilateral under-opercularization (Figure 2c) and enlarged ventricles (Figures 2d and e). In the posterior

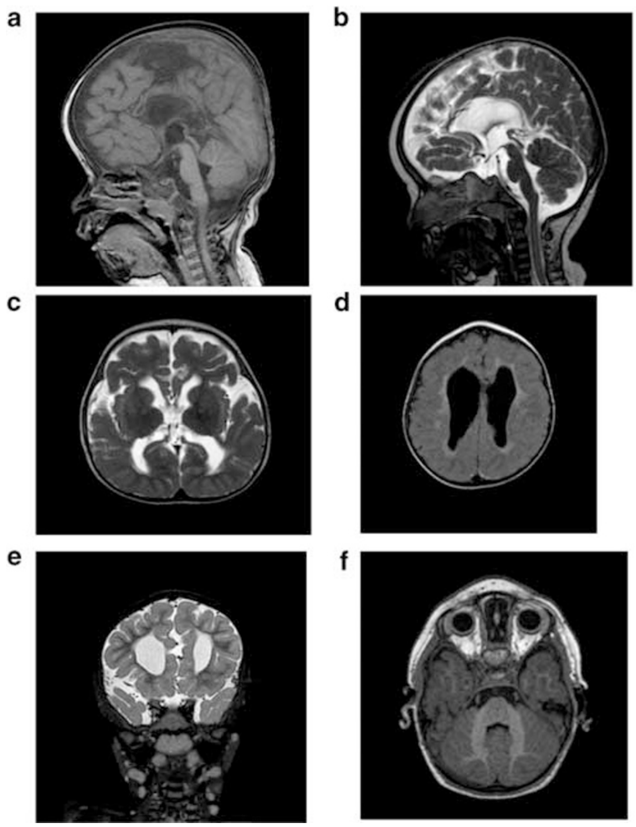

Figure 2 (**a**–**f**) MRI images of two affected individuals, V7 (**a**, T1 sagital; **c**, T2 axial; **e**, T2IR coronal) and IV5 (**b**, T2 sagital; **d**, T2flair axial; **f**, T1 axial), at 1.5 and 2 years, respectively. Note the very thin corpus callosum, generalized brain atrophy and enlarged ventricles.

fossa, the brain stem was relatively small, whereas the vermis was normal in size and shape (Figure 2b). White matter paucity was noted both in the cerebrum and in cerebellar hemispheres (Figures 2d–f).

Linkage analysis

Four affected individuals (IV5, V1, V4, V7 and, Figure 1) and 14 healthy family members (parents and siblings of surviving affected individuals in Figure 1, excluding IV6 and IV7) were available for detailed clinical and mutational analyses. On the basis of consanguinity of the families studied, we assumed that the phenotype was a consequence of a founder effect. We first used polymorphic markers to test affected individuals for homoyozgosity in loci of genes known to be associated with inherited defects of white matter or agenesis of corpus callosum. Affected individuals were shown to be non-homozygous at the loci of *MRPS16*, *SLC12A6*, *SPG11*, *EIF2B1-5*, *GALC*, *GFAP*, *NDUFV1* and *ARSA*, ruling out combined oxidative phosphorylation deficiency-2 (COXPD2 (MIM 610498)), corpus callosum with peripheral neuropathy (ACCPN (MIM 218000)), spastic paraplegia-11 (SPG11 (MIM 604360)), leukoencephalopathy with vanishing white matter (MIM 603896), Krabbe disease (MIM 245200), Alexander disease (MIM 203450) and metachromatic leukodystrophy (MIM 250100; data not shown). We then proceeded to perform genome-wide linkage analysis of four patients, four obligatory carriers (parents of affected individuals) and one healthy sibling (IV1, IV2, V1, V2, IV3, III5, V4, V7 and IV5, depicted in Figure 1), using 250K SNP Arrays (GeneChip Human Mapping 500K Array Set (Affymetrix)) as previously described.[3,5] A single region of homozygosity on chromosome 1p33-1p32.3 was identified, that was common to all affected individuals. Fine mapping[2,6] testing the 18 available DNA samples with polymorphic markers narrowed down the locus to 6.75 cM (7.25 Mb) between *D1S2824* and *D1S200*, with a maximum multipoint LOD score of six calculated using SUPERLINK[4] (data not shown).

Mutation analysis

On the basis of our novel Syndrome to Gene (S2G) software,[8] using *EIF2B1* as a reference gene, the 62 genes within the disease-associated locus were prioritized. No mutation was found in the coding region and flanking intron sequences of the top seven candidate genes (data not shown). However, sequence analysis of the eighth candidate, *DHCR24* (GenBank accession number NM_014762.3), revealed a novel missense mutation c.307C>T in exon 2 (Figure 3), which resulted in substitution of arginine to cysteine at amino acid 103 (p.R103C) adjacent to the flavin adenine dinucleotide (FAD) binding domain of the encoded protein. The mutation abolishes a recognition site of the restriction enzyme AflIII, allowing easy analysis of the entire kindred and controls. Analysis of all 18 DNA samples of the kindred was compatible with the mutation being associated with the disease phenotype, implying full penetrance (data not shown). The mutation was not found in any of 300 chromosomes from ethnically matched controls tested by restriction analysis.

Functional and biochemical assays

DHCR24 encodes an FAD-dependent oxidoreductase expressed in the endoplasmic reticulum membrane, which catalyzes the reduction of the Δ-24 double bond of sterol intermediates during cholesterol biosynthesis. The protein contains a leader sequence that directs it to the endoplasmic reticulum membrane. Figure 3 demonstrates that R103 of *DHCR24*, which is altered in our patients, is extremely well conserved throughout evolution. Missense mutations in this gene have been associated with desmosterolosis. Of the entire kindred, only two fathers (IV1 and IV4, obligatory healthy carriers of the *DHCR24* mutation) and their affected sons (V1 and V7) were willing to undergo biochemical analysis. We thus went on to measure plasma sterol levels, cholesterol and desmosterol concentrations in plasma collected from these four family members. The healthy carriers had normal levels of cholesterol relative to control (Table 2). Their desmosterol levels were also in the same range as the control. However, when calculated as percentage of total sterols, desmosterol in these carriers was about twice that found in the control. The two affected males had slightly lower cholesterol levels than the control and their carrier fathers. This may be because of their age rather than their condition. However, their desmosterol levels were markedly increased when compared to both the control and their carrier fathers. Although desmosterol made up ~0.1% of total sterols in the carrier fathers, the corresponding fraction in their affected sons was 3.4 and 10.1%, respectively. Thus, although affected individuals and carriers had normal cholesterol levels, there were ~120-fold increased levels of plasma desmosterol in affected individuals and 1.5-fold increased levels in carriers, proving deficient activity of 24-dehydrocholesterol reductase.

DISCUSSION

Cholesterol accounts for 99% of all sterols in mammals, and is essential as a major constituent of membranes, a precursor to numerous signaling molecules, and an inducer of the Hedgehog family of morphogens. Cholesterol can be synthesized via two immediate precursors, 7-dehydrocholesterol or desmosterol. The involvement of cholesterol in embryonic development and

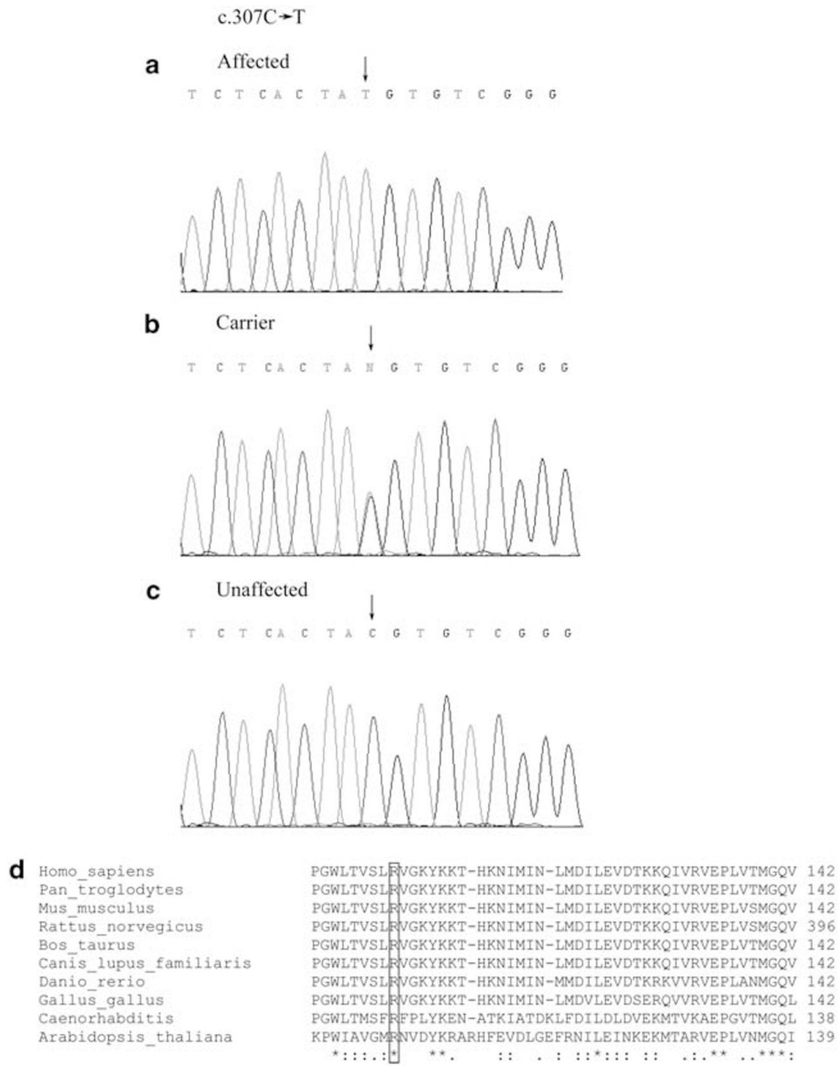

Figure 3 The c.307C>T mutation in exon 2 of *DHCR24.* Sequence analysis is shown for an affected individual (**a**), an obligatory carrier (**b**) and an unaffected individual (**c**). (**d**) ClustalW sequence alignment of human *DHCR24* to orthologs. The c.307C>T mutation (boxed) is in a residue that is highly conserved throughout evolution. Conserved residues are indicated with asterisks.

Table 2 Plasma levels of cholesterol and desmosterol in two male healthy carriers (IV1, IV4) of the *DHCR24* mutation and their two affected sons (V1, V7)

Individual	*Cholesterol (mg/ml)*	*Desmosterol (mg/ml)*	*Desmosterol (% of total sterol fraction)*
V1	1.3	0.045	3.4
V7	1.1	0.126	10.1
IV1	1.8	0.0011	0.1
IV4	1.5	0.0008	0.1
Control	1.8	0.0007	0.04

According to the literature[17] desmosterol levels in plasma of healthy controls are 0.0005 ± 0.0003 mg/ml.

morphogenesis through its role in the hedgehog protein signal transduction pathways provides a potential key to the pathogenesis of cholesterol-associated disorders.[9–13] Human defects in the 7-dehydrocholesterol pathway leading to SLOS (MIM 270400) have been well described. Far less common are human defects in *DHCR24*, encoding the enzyme 3β-hydroxysterol Δ^{24}-reductase, which catalyses the reduction of desmosterol to form cholesterol. To date, two *Dhcr24* null mutant mouse lines have been generated in which cholesterol synthesis is blocked leading to desmosterol accumulation. The lines are on different genetic backgrounds, presenting at first with different phenotypes. *Dhcr24*$^{-/-}$ mice generated by Wechsler *et al*[14] were viable up to adulthood without gross abnormalities, aside from being smaller than their wild-type counterparts and infertile because of the lack of cholesterol derived sex hormones. However, in a later study with a *Dhcr24*$^{-/-}$ mouse strain derived from the strain generated by Wechsler *et al*, most of the mice died prenatally or early postnatally.[7] *Dhcr24*$^{-/-}$ mice generated by Mirza *et al*[15] also died within early postnatal days. Their skin was wrinkleless, movement was restricted and the stomach was always empty. Histological examination of skin revealed features of lethal restrictive dermopathy.

To date, only two clinical cases of desmosterolosis (MIM 602398) have been described, and *DHCR24* mutations of both alleles were demonstrated in both cases.[1] The phenotypes of the two cases are very different, leaving the question of the desmosterolosis phenotype unresolved. FitzPatrick *et al*[16] reported a case of an infant with multiple lethal congenital malformations in whom there was generalized accumulation of desmosterol and relative deficiency of cholesterol. The newborn (born week 34, died shortly after birth) had macrocephaly, hypoplastic nasal bridge, thick alveolar ridges, gingival nodules, cleft palate, total anomalous pulmonary venous drainage, ambiguous genitalia, short limbs and generalized osteosclerosis. The brain showed an immature gyral pattern with poor development of the corpus callosum and gross dilatation of the ventricles. The frontal lobes were disproportionately large and firm and the occitpital lobes were small. Abnormal accumulation of desmosterol was demonstrated in the kidney, liver and brain. Higher than normal levels of the same sterol were detected in plasma samples obtained from both parents.

The only other case of desmosterolosis in the literature[17] is of a boy (born at term) with microcephaly, agenesis of the corpus callosum, downslanting palpebral fissures, bilateral epicanthal folds, submucous cleft palate, micrognathia, mild contractures of the hands, bilateral clubfeet, cutis aplasia and persistent patent ductus arteriosus. At 40 months of age, he was severely developmentally delayed and had failure to thrive. Radiological examination disclosed neither rhizomelic shortness nor osteosclerosis. Plasma sterol quantification at 2 years of age demonstrated normal cholesterol levels but a 100-fold increase in desmosterol. Both parents had mildly increased levels of desmosterol in plasma, consistent with heterozygosity for DHCR24 deficiency. Analysis of sterol metabolism in cultured transformed lymphoblasts showed a 100-fold increased level of desmosterol and a moderately decreased level of cholesterol in the cells the of the patient and a 10-fold elevation of desmosterol in the cells of the mother.

The phenotype of the four cases presented here is consistent and thus establishes a clear human desmosterolosis phenotype. Similar to the case described by Andersson *et al*,[17] our patients had microcephaly with agenesis of corpus callosum, as well as failure to thrive. Most of the affected individuals had also convulsions, nystagmus and strabismus, and micrognathia as well as mild-to-severe contractures of the hands (Table 1). However, other features seen in the previous report (downslanting palpebral fissures, bilateral epicanthal folds, submucous cleft palate, bilateral clubfeet, cutis aplasia and patent ductus arteriosus) were absent in our patients, and are thus not essential features of the desmosterolosis phenotype. The case reported by FitzPatrick *et al*[16] has some features in common with the phenotype we report. However, its clinical presentation is far more severe, either because of the higher desmosterol levels, or representing a wider defect beyond desmosterolosis.

The novel homozygous *DHCR24* mutation found in the kindred we describe is a missense mutation in an extremely conserved amino acid that is immediately adjacent to the FAD-binding domain of the protein. The homozygous mutation in the case of Andersson *et al*[17] is in a less conserved amino acid that is within the same domain, perhaps explaining the partial similarity in the phenotype. In contrast, compound heterozygous mutations seen in the phenotypically less similar case described by FitzPatrick *et al*[16] are in amino acids that are far less conserved and that are remote from the FAD-binding domain. It remains unclear whether the case of FitzPatrick *et al* has a phenotype different than the other cases only because of the dissimilar mutations, or because the patient had additional unidentified molecular defects. It is of interest to note that in another cholesterol pathway disease, SLOS, various mutations in the same gene cause a large scope of clinical phenotypes: severe biochemical disorder causes a lethal malformation syndrome, whereas milder missense mutations can present as minimally dysmorphic children with learning disability.[18] As suggested by Andersson *et al*,[17] the central nervous system anomalies seen in desmostrolerosis may be due to impaired sonic hedgehog (SHH) signaling which is known to require adequate cellular levels of cholesterol for normal function. The phenotypic variability of desmosterolosis might thus be due to different degrees of perturbations of the SHH pathway.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

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WEB RESOURCES

Chromas: http://www.technelysium.com.au/chromas.html
Conseq server: http://conseq.bioinfo.tau.ac.il/
HaploPainter: http://haplopainter.sourceforge.net/index.html
NEBcutter V2.0: http://tools.neb.com/NEBcutter2/
Online Mendelian Inheritance in Man (OMIM): http://www.ncbi.nlm.nih.gov/Omim/
Primer3, (v. 0.4.0) Pick primers from a DNA sequence: http://frodo.wi.mit.edu/primer3/
Simple Modular Architecture Research Tool (SMART): http://smart.embl-heidelberg.de/
Superlink online version 1.5: http://bioinfo.cs.technion.ac.il/superlink-online/
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