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Discordant Clinical Phenotype and Sterol Biochemistry in Smith-Lemli-Opitz Syndrome

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Abstract

Smith-Lemli-Opitz syndrome (SLOS) is a multiple malformation syndrome resulting from mutations of the 7-dehydrocholesterol reductase gene (*DHCR7*). During *de novo* cholesterol biosynthesis, *DHCR7* catalyzes the conversion of 7-dehydrocholesterol (7DHC) to cholesterol. A clinical diagnosis of SLOS is confirmed biochemically by the presence of elevated levels of 7-dehydrocholesterol. Phenotypic severity of SLOS has previously been shown to correlate with the 7DHC/cholesterol ratio. In this case report, we describe a patient with a severe SLOS phenotype, but a very low serum 7DHC/cholesterol ratio. We further demonstrate that this discordance is due to alternative splicing of a previously unreported IVS5+3 A>T mutation. This mutation results in the transcription of both normal and mutant mRNA transcripts. We postulate that alternative splicing of the IVS5+3 A>T results in insufficient *DHCR7* activity during embryogenesis, but sufficient *DHCR7* activity once cholesterol synthetic rates decrease postnatally. This case is a unique observation that underscores the adjunctive use of fibroblast and molecular testing in ambiguous cases of SLOS and may provide insight into the potential efficacy of therapeutic interventions altering postnatal cholesterol biosynthesis.

Keywords

Smith-Lemli-Opitz syndrome; SLOS; malformations; 7-dehydrocholesterol (7DHC); cholesterol; inborn errors

Introduction

Smith-Lemli-Opitz syndrome (SLOS, OMIM #270400) is an autosomal recessive, multiple malformation syndrome due to mutations of the 7-dehydrocholesterol reductase (*DHCR7*) gene (Fitzky *et al.* 1998; Wassif *et al.* 1998; Waterham *et al.* 1998). *DHCR7* reduces 7-dehydrocholesterol to cholesterol in the final step of the Kandutsch-Russell cholesterol synthesis pathway (Kandutsch and Russell 1960). The impairment of *DHCR7* typically results in elevated 7DHC, and its isomer 8DHC, and low cholesterol in patients with classical to severe phenotypes. This combination of increased precursor sterols and low cholesterol values likely has a direct impact on development. Cholesterol is critical for the maturation of the hedgehog family of morphogens. Cooper *et al.* (Cooper *et al.* 2003) demonstrated that reduced total sterol levels in SLOS disrupt the hedgehog signaling pathway at the level of Smoothen. Physical findings in SLOS include microcephaly, micrognathia, cleft palate, second and third toe syndactyly, polydactyly, cardiac

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malformations, pyloric stenosis, and genital malformations (Porter 2008). These abnormalities are compounded by developmental delay, often being labeled “failure to thrive”, mental retardation, and behavioral problems. The phenotypic spectrum includes autistic symptoms (Tierney *et al.* 2001).

The phenotypic presentation of SLOS varies broadly. The number and severity of congenital malformations seen in SLOS is inversely correlated to the ratio of dehydrosterols as a function of total sterols (Jira *et al.* 2001; Witsch-Baumgartner *et al.* 2000). An inverse correlation has also been observed between residual DHCR7 activity and phenotypic severity (Wassif *et al.* 2005). As one would expect, subjects with low DHCR7 activity tend to have higher 7DHC and lower cholesterol levels. Severely affected patients frequently have little or no measurable residual DHCR7 activity and dehydrocholesterol/total sterol ratios exceeding 50%. In contrast, mild to classical patients have significant residual DHCR7 function (Wassif *et al.* 2005).

The c.964-1G>C (IVS8-1 G>C) allele is the most common mutation, (Battaile *et al.* 2001; Correa-Cerro and Porter 2005; Waye *et al.* 2002; Wright *et al.* 2003) accounting for about a third of ascertained alleles. IVS8-1 G>C is a splice acceptor mutation and is a null mutation. In the homozygous state or in association with another null mutation, IVS8-1 G>C results in a severe phenotype (Wassif *et al.* 1998; Witsch-Baumgartner *et al.* 2000). The majority of SLOS patients are compound heterozygotes. IVS8-1 G>C in combination with a missense allele encoding a protein with residual enzymatic function is frequently observed in mild to classical cases.

In this paper, we report a case where the patient presented with a multitude of congenital anomalies consistent with a severe SLOS phenotype but discordant diagnostic serum sterol levels. Cholesterol levels were normal, and 7DHC levels were minimally increased. *DHCR7* genotyping and analysis of sterol synthesis in fibroblasts confirm the clinical diagnosis. To explain this discordance, we postulated that a low level of correct splicing, resulting from a previously unreported IVS5+3 A>T splice donor mutation, provided insufficient DHCR7 activity during embryogenesis but sufficient postnatal DHCR7 activity. This case underscores the use of molecular and fibroblast testing in the diagnosis of SLOS and may provide future insight into the potential efficacy of postnatal therapy on brain development.

Clinical Report

This female patient, the product of nonconsanguineous Caucasian parents, was born prematurely at 34 6/7 weeks of gestation. The mother was a multigravida 27-year-old with a prior history of one spontaneous abortion, and the father was 26 years old. The pregnancy was complicated by a prenatal diagnosis of an atrioventricular canal defect. Triple screen was normal. Birth was by emergent Cesarean section performed due to reduced fetal movement, severe oligohydramnios, and intrauterine growth retardation. Birthweight was 1790g (5th centile), length 38cm (<3rd centile), and head circumference 28cm (3rd centile). Multiple malformations consistent with SLOS were noted at birth. These included microcephaly, micrognathia, cleft soft palate, bilateral postaxial polydactyly of the hands, and syndactyly of the second and third toes. This patient also had a thyroglossal duct cyst, a finding not previously reported in SLOS. The cleft palate was repaired at 2 months of age. A gastrostomy tube was placed for nutritional support at 3 ½ months of age. The child underwent ligation of a patent ductus arteriosus at six days of age, and the atrioventricular canal defect with a moderate atrial and small ventricular defect was repaired at 11 months. Central nervous system malformations included a Chiari malformation seen on MRI at six months of age, and a tethered spinal cord was diagnosed by ultrasound and MRI and surgically released at 7 months of age. Right renal agenesis was diagnosed by ultrasound,

and there was grade three vesicoureteral reflux on the left. An anoplasty was performed at eight months of age for anal stenosis. In addition to the aforementioned limb defects, the child had bilateral hip dysplasia, and the left hip was successfully reduced at 3 ½ months of age. Correction of bilateral valgus deformity of the ankles was attempted at 9 months.

The patient presented to us at 12 months of age. Weight was 6230g (<3rd centile), length 63.5cm (<3rd centile), and head circumference 41cm (<3rd centile). Cranial features included microcephaly, plagiocephaly, and a broad forehead. Dysmorphic facial features included a broad flat nasal bridge, and relative hypertelorism with an inner canthal distance of 2.5 cm (50th centile). The outer canthal and interpupillary distances were 6.6 cm (15th centile) and 4.4 cm (30th centile), respectively. Bilateral ptosis and partial epicanthal folds were present. The nose was small with anteverted nares. The oral exam was notable for micrognathia, a high arched palate with a U-shaped cleft, broad alveolar ridges, microglossia, and cyst-like nodules beneath the tongue. Ears were low set and had a slight posterior rotation. Nipples were wide-spaced and low set, and a vertical surgical scar was present. The abdominal exam was notable only for the gastrostomy tube, and the genital exam was unremarkable. Diffuse cutis marmorata was noted. The upper extremities appeared short relative to the body, and examination of the hands was notable for bilateral brachydactyly, bilateral postaxial polydactyly with a well formed sixth digit, multi component single palmar crease, and retention of the fetal finger pads. Examination of the lower extremity was notable for bilateral valgus deformity of the ankles and bilateral syndactyly of the second and third toes. Neurological exam was notable for marked hypotonia but otherwise was age appropriate. Using the phenotypic severity scale initially developed by Bialer (Bialer *et al.* 1987) and subsequently modified by Kelley (Kratz and Kelley 1999), this patient has a phenotypic severity score of 50.

Despite the major malformations and history of multiple medical interventions, this child's neurological delay was significantly less than expected. Although being in a spica cast for 11 weeks, she rolled from side to side at 7 months of age and began to stand with assistance at 11 months. At 9 months, she could lift her head, reach for objects, and transfer objects from hand-to-hand. She could also imitate sounds. She sat with support for 2 minutes at 10 months of age. At 12 months of age, she was not yet crawling or walking independently.

Methods and Results

Human Subjects Research

This patient was enrolled in a clinical protocol approved by the NICHD Institutional Review Board, and informed consent was obtained.

Molecular analysis

Karyotype was 46, XX, and FISH analysis excluded DiGeorge syndrome. Array CGH was normal (GeneDx). Sterol testing was performed at 4 and 6 months of age. Serum 7DHC levels were 5.68 mcg/ml and undetectable, respectively. Upon evaluation at 12 months, serum cholesterol levels were 115mg/dl, and 7DHC was 0.67 mcg/ml. Normal range for 7DHC is 0.16 +/- 0.09 mcg/ml (Kelley 1995). *DHCR7* mutation analysis was performed by GeneDx. Genotype was IVS8-1G>C:IVS5+3A>T.

The patient and control fibroblasts were plated at a density of 650,000 cells per T75 flask and cultured in 7.5% lipoprotein deficient serum (LPDS) for 5 days. mRNA was isolated, and RT-PCR was performed to obtain *DHCR7* cDNA (Krakowiak *et al.* 2000). Three prominent bands, labeled A, B, and C, were observed in the proband compared to the single expected band present in the control (Figure 1A). Band A was approximately 1850 nucleotides in length, consistent with the known 134 base pair insertion associated with the

IVS8-1 G>C splice acceptor mutation (Wassif *et al.* 1998). This was confirmed by sequencing. Band B was similar in size to that observed from control cells (1690 nucleotides, lane 2), and sequence analysis showed a normal *DHCR7* transcript. Band C is 1590 nucleotides in length, consistent with a deletion. Sequencing of band C revealed the absence of exon 5 (Figure 1B). This alternative splicing, or exon skipping, resulted in the loss of 91 bases of exon 5 within the cDNA and a single -1 base frameshift in the coding sequence of exon 6. The predicted protein resulting from translation of this deleted transcript will have a leucine residue inserted at amino acid position 109 followed by premature truncation (Figure 1B).

***In vitro* sterol analysis**

The proband's fibroblasts were cultured in LPDS for 5 days, and sterol analysis was performed by gas chromatography/mass spectrometry (GC-MS) as previously described (Wassif *et al.* 2005). Protein determinations were done using Bio-Rad's Micro DC Protein Assay as per manufacturer's protocol. As shown in figure 1D, we observed a significant increase in both 7DHC (5.6 mcg/mg protein) and average 7DHC fraction (34%) in patient fibroblasts. This fractional DHC value is similar to values seen from fibroblasts isolated from classical SLOS patients (Krakowiak *et al.* 2000).

Comparison of plasma sterol levels with other SLOS patients

Table 1 demonstrates how divergence of this patient's phenotype and biochemistry are in comparison to other SLOS patients. Out of a total of 47 SLOS patients, we have evaluated five patients with diagnostic serum 7DHC values ≤ 10 mcg/ml. In this group of patients, the mean plasma cholesterol and 7DHC levels were 131.4 ± 25.7 mg/dl and 2.1 ± 1.4 mcg/ml respectively. Mean 7DHC/cholesterol ratio was $0.16 \pm 0.54\%$. These values are similar to those observed in the current patient (115 mg/dl, 0.67 mcg/ml, and 0.06% respectively), but stand in marked contrast to a score of 50 when these other patients had a mean severity score of 8.2 ± 4.1 . We have also evaluated six other patients with severity scores ≥ 39 . In this group of patients mean cholesterol, 7DHC, 7DHC/cholesterol ratio, and severity score were 16.9 ± 11.8 mg/dl, 200 ± 159 mcg/ml, $129 \pm 88\%$, and 47.8 ± 12.7 , respectively. With a severity score of 50, this patient's phenotype is consistent with this cohort of patients; however, her plasma sterol values stand in marked contrast.

Discussion

Typically, the phenotypic severity of SLOS patients correlates with the severity of the *DHCR7* deficiency. In this case, the phenotype and serum sterol profile are markedly discordant in comparison to other cases of SLOS (Table 1). While she presents with a severe phenotype, her serum 7DHC values are consistent with what is typically observed in mild cases. *DHCR7* genotyping identified the common IVS8-1 G>C mutation and a novel splice donor IVS5+3 A>T mutation. Since the IVS8-1 G>C mutation is a null allele, we evaluated the IVS5+3A>T mutation to explain this discordance.

As our data demonstrates, the IVS5+3A>T allele produces both a normal transcript and an alternatively spliced transcript predicted to encode a truncated protein. Therefore, the patient has some level of normal functioning *DHCR7*. During embryonic development, the biological need for cholesterol is high, and de novo cholesterol synthesis by the fetus is required. The multiple congenital malformations observed in this case suggest that *DHCR7* activity was insufficient to meet the cholesterol demand. Following birth, the demand for cholesterol decreases, and dietary cholesterol becomes available. In our patient, postnatal level of functioning *DHCR7* appears to be sufficient for this lower cholesterol synthetic requirement. This would explain the very small elevation in serum 7DHC and normal

cholesterol levels. Our *in vitro* fibroblast experiments support this hypothesis. During growth in cholesterol deficient medium, the intrinsic biochemical defect becomes readily apparent. Although we think that this is a plausible explanation, an alternative explanation would be that the ratio of correctly to incorrectly spliced *DHCR7* transcript varies in embryonic and mature tissues.

This case underscores the diagnostic utility of evaluating sterol synthesis *in vitro* and obtaining *DHCR7* mutational analysis in cases when plasma sterol testing is ambiguous (Porter 2008). These situations involve cases with a very mild phenotype or false positives due to treatment with psychiatric drugs. This case also illustrates the need for additional evaluation when the phenotype is consistent with SLOS but the diagnostic sterols are negative, such as found at 6 months in this child.

An interesting question for the future will be the status of this child's mental development. Neurological defects in SLOS are likely to be a combination of both fixed developmental defects and functional defects due to abnormal sterol biochemistry in synapses, neuronal membranes, and myelin. Functional problems due to abnormal sterol biochemistry may be amendable to therapeutic interventions that can increase residual *DHCR7* function from hypomorphic alleles. A similar situation exists in the *Dhcr7*^{T93M} mutant mouse. High levels of expression of the *Dhcr7*^{T93M} allele result in spontaneous biochemical correction (Correa-Cerro *et al.* 2006). We have shown that increased expression of hypomorphic *DHCR7* alleles induced by simvastatin therapy increase cholesterol synthesis both in human fibroblasts (Wassif *et al.* 2005) and mouse tissues (Correa-Cerro *et al.* 2006). The degree of this child's developmental delay is much less than expected given the phenotypic severity. An observation that is consistent with the idea that functional deficits can be ameliorated by increasing endogenous central nervous system cholesterol synthesis in SLOS patients.

Acknowledgments

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Figure 1. Molecular and biochemical characterization

A) RT-PCR analysis demonstrated the presence of multiple bands in the proband. Lane 1: 1636 bp and 2036 bp standards. Lane 2: *DHCR7* RT-PCR analysis of a control cell line demonstrating the expected 1690 bp product. Lane 3: *DHCR7* RT-PCR analysis of the proband's cells. Three prominent bands of 1590 bp (A), 1690 bp (B) and 1850 bp (C) were observed. **B)** *DHCR7* sequence analysis of band B (panel A) and band C (panel B). Sequencing of band B showed normal splicing of exons 4 and 5. In contrast sequencing of band C showed deletion of exon 5. Deletion of exon 5 results in the predicted inclusion of a leucine residue (boxed) and a premature termination codon (*). GC-MS sterol analysis of proband's plasma (**C**) and cells grown in cholesterol deficient medium (**D**). Total ion chromatograms are shown. Peak 1 is the internal standard coprostanol. Peaks 2 and 3 are cholesterol and 7DHC, respectively.

Table 1

Comparison of sterol biochemistry and phenotypic severity score for the proband and other SLOS subjects.

Patient Number	7DHC (ug/ml)	Cholesterol (mg/dl)	Severity Score	Percent 7DHC/Cholesterol
1	2.04	142	11	0.14
2	4.42	88	11	0.50
3	2.02	131	11	0.15
4	1.1	155	2	0.07
5	0.96	141	6	0.07
6	0.67	115	50	0.06
7	254	28	56	90.71
8	237	8	39	296.25
9	476	35	39	136.00
10	112	8.8	44	127.27
11	74	14	39	52.86
12	52	7.6	70	68.42