Published in final edited form as:

Am J Perinatol. 2018 August ; 35(10): 936–939. doi:10.1055/s-0038-1626705.

Smith-Lemli-Opitz Mutations in Unexplained Stillbirths

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Abstract

Objective—Smith-Lemli-Opitz syndrome (SLOS) is an autosomal recessive syndrome caused by a defect in cholesterol biosynthesis with mutations in 7-dehydrosterol reductase (DHCR7). 3% of Caucasians carry DHCR7 mutations, theoretically resulting in a homozygote frequency of 1/4000. However, SLOS occurs in only 1/20,000–60,000 live births. Our objective was to assess DHCR7 mutations in unexplained stillbirths.

Study Design—Prospective, multicenter, population-based case-control study of all stillbirths and a representative sample of live births enrolled in 5 geographic areas. Cases with stillbirth due to obstetric complications, infection, or aneuploidy, and those with poor quality DNA were excluded. DNA was extracted from placental tissue stored at – 80°C, and exons 3–9 of the DHCR7 gene were amplified, purified, and subjected to bidirectional sequencing to identify mutations.

Results—144 stillbirths were unexplained and had adequate DNA for analysis. Nine stillbirths of 139 (6.5%) had a single mutation in one allele in coding exons 3–9 of DHCR7 (Table 1). One case (0.7%) was a compound heterozygote for mutations in exons 3–9 of DHCR7; this fetus had no clinical or histologic features of SLOS.

Conclusion—We detected SLOS mutations in only 0.7% of stillbirths. This does not support a strong association between unrecognized DHCR7 mutations and stillbirth.

Keywords

Smith-Lemli-Opitz; stillbirth; genetic syndromes; cholesterol synthesis

Introduction

Smith-Lemli-Opitz syndrome (SLOS) is an autosomal recessive genetic syndrome due to a defect in cholesterol biosynthesis occurring in approximately 1/20,000-60,000 live births¹⁻⁵. The syndrome is characterized by multiple congenital malformations including cleft palate, microcephaly, large external ears, broad alveolar ridges, postaxial polydactyly, and increased whorl patterns on the fingertips. Pronounced growth restriction and moderate to severe intellectual disability are also part of the syndrome. The phenotype is highly variable; type I is a mild form and type II is more severe.

SLOS is caused by mutations in the delta-7-dehydrocholesterol reductase (DHCR7) gene⁶. This gene is on chromosome region 11q13 and codes for the terminal enzyme crucial to cholesterol biosynthesis. There are at least 154 known mutations; IVS8-1G→C, T93M, and W151X are three of the most common⁶⁻¹⁰. Different mutations confer different phenotypes and prognosis¹¹, and clinical phenotypes correlate reasonably well, albeit imperfectly with the ability of lymphoblasts to synthesize cholesterol¹².

Carrier frequency of DHCR7 mutations is high. An Ontario based study of 2,978 people found 24 carriers of IVS8-1G→C mutation⁵. Using the detected rate of IVS8-1G→C mutation in the general population and the frequency of IVS8-1G→C mutation found in individuals with SLOS, the estimated carrier frequency for all mutations is 1 in 30¹³. Given this high carrier frequency for DHCR7 mutation, we would expect SLOS to occur in 1:5,900-1:13,500 births. However, SLOS occurs in only 1:20,000-60,000 live births⁵. This suggests that SLOS may account for a number of stillbirths and early pregnancy losses since the prevalence is much lower than expected in the live birth population. Thus, our objective was to explore the frequency of SLOS mutations in well-characterized stillbirths.

Methods

This is a secondary analysis of the *Eunice Kennedy Shriver* National Institute of Child Health and Human Development Stillbirth Collaborative Research Network study¹⁴. The SCRNC was a prospective, multicenter, population based cohort with attempts to include all stillbirths greater than or equal to 20 weeks gestation and a representative sample of live births. Five geographic areas (Rhode Island, portions of Massachusetts, Georgia, Texas, and Utah) were included as defined by county lines, including 59 hospitals averaging more than 80,000 deliveries per year to area residents. Participants were enrolled at delivery between March 2006 and September 2008.

Study design, methodology, and sample size calculations have been previously published^{14,15}. For this analysis, only stillbirths were included. Stillbirths were defined as births at or after 20 weeks' gestation with Apgar scores of 0 at 1 and 5 minutes and no signs of life on direct observation. Fetal deaths at 18 or 19 weeks' gestation without good dating criteria were also included to ensure all potential cases at or after 20 weeks' gestation were studied. Gestational age was determined using data from assisted reproductive technology, first day of last menstrual period, and ultrasound¹⁴. Deliveries resulting from termination of a live fetus were excluded. The study was approved by the institutional review boards of each

clinical site and the Data-Coordinating and Analysis Center, and all mothers gave written informed consent. For this analysis, participants with stillbirth due to obstetric complications, infections, or aneuploidy were excluded. Cause of stillbirth was determined using the Initial Causes of Fetal Death (INCODE) system (Dudley 2010). Cases with only poor quality DNA available were also excluded. As this was an exploratory study, there were no pre-specified sample size calculations.

DNA was isolated from a section of placenta obtained from stillbirths and stored at -80°C as previously described (Parker methods). The extraction was completed using the Pure Gene DNA Purification System (Qiagen, Valencia, CA) per the manufacturer's protocols. Concentration and yield were determined using a Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, DE). Samples were normalized to a standard concentration.

Primers were designed to amplify exons 3-9 of the DCHR7 gene. 5' and 3' flanking regions of each exon were also amplified to detect possible splice-site mutations. Exon 9 was amplified in two segments due to the size of the exon. Generally, 25 ng of DNA was amplified in a reaction containing 2.0-3.0 mM MgCl_2 , 0.2 mM dNTPs, 0.5uM of each primer, and 1.25-2.5 units of Amplitaq Gold (Life Technologies, Foster, City, CA). Amplification was done on an ABI 9700 thermal cycler (Life Technologies, Foster, City, CA) for 35 cycles at annealing temperatures between 55-60°C. After PCR, samples were purified to remove excess primers and enzymes prior to BigDye Terminator cycle sequencing. Cleanup was completed using ExoSap. (Affymetrix, Santa Clara, CA). Samples were quantified by gel electrophoresis and PICO Green quantitation.

Samples underwent bidirectional cycle sequencing using the Big Dye Terminator v3.1 kit (Life Technologies, Foster, City, CA) per manufacturer's protocols. 30ng of sample from each exon was combined with 4 pmoles of the exon specific primer. Both forward and reverse reactions were prepared. Samples were then purified using Agencourt Amp Pure magnetic beads and manufacture's protocols (Beckman Coulter, Brea, CA). Fluorescent sequencing was completed on an ABI 3730XL capillary electrophoresis instrument (Life Technologies, Foster, City, CA). Samples were electrophoresed for 2 hours.

Sequences were analyzed using Sequencer 4.9 software (Gene Codes, Ann Arbor, MI). Variants were examined for exons 3-9 along with 20 base pairs 5' and 3' of the exon.

Results

There were 663 enrolled stillbirth pregnancies in the original cohort, of which 512 in 500 women had complete postmortem examinations. We then excluded 312 cases with a probable cause of death based on INCODE (obstetric condition, placental abnormality, fetal genetic/structural abnormality, infection, umbilical cord abnormality, hypertensive condition, other maternal medical condition), as well as 5 multiple gestations. Of the remainder, extracted DNA was of sufficient quality for 144 potential cases. Of these 144, 73 were white, 46 Hispanic, 17 black, and 8 of other race. 139/144 (97%) yielded informative DNA assays. Nine stillbirths of 139 (6.5) had a single mutation in one allele in coding exons 3-9

of DHCR7 (Table 1). These nine stillbirths with DHCR7 mutations included 4/73 (5.5%) white stillbirths, 3/46 (6.5%) Hispanic stillbirths, and 2/17 (11.8%) black stillbirths. One Hispanic case was a compound heterozygote for mutations in exons 3-9 of DHCR7; the remaining eight cases were single heterozygous mutations for the mutation and thus would not be clinically affected. The one case of compound heterozygosity had no clinical or histologic features of SLO and was unexplained. There were no homozygous cases.

There were 15 apparently benign polymorphisms in exon 4 position 240 T77, including two homozygous cases. There were no cases of the most common mutation in Caucasians: IVS8-1G>C.

Discussion

Only 1 out of the 139 stillbirths with informative DNA analysis in women without noted cause of death by INCODE had two DHCR7 mutations, consistent with a possible SLOS diagnosis. Thus, the rate of SLOS based on mutation analysis was 0.7% in this stillbirth population. It is noteworthy that this case did not have any phenotypic features of SLOS.

This rate is less than what would be expected given the discrepancy in the rate among live births and the carrier frequency. The carrier frequency of SLOS mutations is 1/30 to 1/124 in European and North American populations, as high as 3.3%^{5,13}. Accordingly, one would anticipate a live birth rate as high as 0.03%. In contrast, the observed rate of SLOS in live births is 0.002-0.005%⁵. Thus, one might presume that the remaining “missing” fetuses with SLOS are stillborn. In 2014 there were 3,988,076 births¹⁶ in the U.S. If 200 of those births (using 0.005% frequency of SLOS in live births) had SLOS, there would remain 996 “missing” or unaccounted for SLOS fetuses because the carrier frequency of DHCR7 is 1/30. In 2013, there were 23,595 fetal deaths at 20 weeks or more¹⁷. If the difference between observed and expected SLOS rates in live births was due to affected fetuses dying prior to birth, one would anticipate 996 out of those 23,595 stillbirths to be affected by SLOS, or 4.2%. Our finding of SLOS in 0.7% of stillbirths is much lower than this anticipated rate.

There are several possible reasons for our findings. One is that the carrier frequency for DHCR7 is population dependent and less common than reported in some studies. A recent analysis from the 1,000 Genomes Project found a carrier frequency of only 1.01% in the general population instead of the previously reported 1/30 or 3.3%¹⁸. This frequency is more consistent with an SLOS disease frequency of 1/39,215 live births, which is consistent with what is often reported. The carrier frequency in our stillbirth population was 6.4% (1/15-1/16), which is higher than the previously reported 1/30. However, it is not clear how simply being a carrier for DHCR7 mutation would confer increased stillbirth risk.

Another possibility is that SLOS is associated with early pregnancy loss. Our study only evaluated late second and third trimester losses. Since SLOS is present in live births and in some cases is associated with mild phenotypes, we postulated it would be more likely to be associated with late gestation losses. To our knowledge, SLOS had not been systematically

assessed in early pregnancy losses. The burden of SLOS mutations in early fetal losses between 10 and 20 weeks also remains unknown.

Prenatal diagnosis of SLOS is possible by using amniocentesis or chorionic villus sampling to assay DHCR7 mutations and 7-dehydrocholesterol and 7-dehydrocholesterol/cholesterol ratios¹⁹. This is typically done when SLOS is suspected based on abnormal findings on obstetric sonogram or in families with known mutations. In addition, low levels of unconjugated estriol on maternal serum marker screen are associated with an increase in the risk for SLOS²⁰. Amniocentesis or chorionic villus sampling (CVS) can be used to test for elevated levels of 7-dehydrocholesterol due to deficient DHCR7^{6,19,21}. CVS has been used to evaluate DHCR7 activity in vitro²². If there is a known familial DHCR7 mutation, this can be tested by amniocentesis or CVS as well²³. However, population based diagnostic testing is not realistic given the selective population currently undergoing amniocentesis and CVS. It would be possible to evaluate early pregnancy loss specimens for genetic evidence of overrepresented mutations of the DHCR7 gene. As the cost of widespread screening decreases, assessment of more asymptomatic people will clarify the true rates of DHCR7 mutations and associated phenotypes.

A limitation of our study is the small sample size. We were only able to screen a limited number of cases due to the expense of targeted gene sequencing. Accordingly, we cannot give precise estimates of the contribution of SLOS to stillbirth other than to state that it is sporadic and does not appear to be common in unexplained stillbirth. In addition, our population tested may be genetically different from these previously reported groups. Rates of SLOS and DHCR7 mutations vary by race and ethnicity^{5,13}. We assessed a range of races and ethnicities including a large number of non-Hispanic whites (with the highest rate of mutations) in this exploratory study. Also, there may have been some bias in which cases had samples with high quality DNA, or SLOS may have been present in cases that were apparently “explained.” Finally, it would be ideal to assess contemporaneous controls (live births) for DHCR7 mutations.

Our study has several strengths as well. It is the largest number of stillbirth cases to be systematically evaluated for SLOS mutations (MEDLINE search). Cases of stillbirth were well characterized and those with clear indications were excluded. Our cases were ethnically and racially diverse and SLOS genes were comprehensively evaluated.

Although sporadic stillbirths are associated with SLOS²⁴, our data detected homozygous mutations consistent with SLOS in only 0.7% of stillbirths. This does not support a strong association between unrecognized DHCR7 mutations and stillbirth. Our data do not support routine genetic testing for SLOS as part of a routine evaluation for stillbirth, unless there are clinical features concerning for SLOS.

Acknowledgments

Funding: Supported by grants (HD45925, HD45944, HD45952, HD45953, HD45954, and HD45925) from the Eunice Kennedy Shriver National Institute of Child Health and Human Development

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Table 1
DHCR7 Mutations in Stillbirths by Race

DHCR7 Exon/Mutation	White	Black	Hispanic	Total
Exon 3 I 9 F	0	0	1	1
Exon 4 IV3-4	0	1	1	2
Exon 4 Q63Q	0	0	0	0
Exon 4 T69T	0	0	0	0
Exon 4 T77T*	9	3	2	14
Exon 4 IVS4 + 22	0	0	1	1
Exon 5	0	0	0	0
Exon 6 N146N	0	0	0	0
Exon 6 L157P	1	0	0	1
Exon 6 A190A	0	1	0	1
Exon 6 T193T	1	0	0	1
Exon 6 IVS6 + 15	0	0	0	0
Exon 7 N252Y	2	0	1	3
Exon 7 L272L	0	0	0	0
Exon 8	0	0	0	0
Exon 9A	0	0	0	0
Exon 9B D386D	0	0	0	0
Exon 9B G425G	0	0	0	0
Total	13	5	6	24
Total Deleterious Mutations	4	2	4	10

* benign polymorphism