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Mutational Spectrum in the Δ7-Sterol Reductase Gene and Genotype-Phenotype Correlation in 84 Patients with Smith-Lemli-Opitz Syndrome

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Summary

Smith-Lemli-Opitz syndrome (SLOS), an autosomal recessive malformation syndrome, ranges in clinical severity from mild dysmorphism and moderate mental retardation to severe congenital malformation and intrauterine lethality. Mutations in the gene for Δ7-sterol reductase (*DHCR7*), which catalyzes the final step in cholesterol biosynthesis in the endoplasmic reticulum (ER), cause SLOS. We have determined, in 84 patients with clinically and biochemically characterized SLOS (detection rate 96%), the mutational spectrum in the *DHCR7* gene. Forty different SLOS mutations, some frequent, were identified. On the basis of mutation type and expression studies in the HEK293-derived cell line tsA-201, we grouped mutations into four classes: nonsense and splice-site mutations resulting in putative null alleles, missense mutations in the transmembrane domains (TM), mutations in the 4th cytoplasmic loop (4L), and mutations in the C-terminal ER domain (CT). All but one of the tested missense mutations reduced protein stability. Concentrations of the cholesterol precursor 7-dehydrocholesterol and clinical severity scores correlated with mutation classes. The mildest clinical phenotypes were associated with TM and CT mutations, and the most severe types were associated with 0 and 4L mutations. Most homozygotes for null alleles had severe SLOS; one patient had a moderate phenotype. Homozygosity for 0 mutations in *DHCR7* appears compatible with life, suggesting that cholesterol may be synthesized in the absence of this enzyme or that exogenous sources of cholesterol can be used.

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Introduction

Smith-Lemli-Opitz syndrome (SLOS [MIM 270400]) (Smith et al. 1964) is an autosomal recessive metabolic disorder that causes variable congenital anomalies, including microcephaly, cleft palate, syndactyly of toes 2/3, polydactyly, visceral malformations, variable structural anomalies of the heart, ambiguous genitalia in males, failure to thrive, and mental retardation. SLOS is a relatively common disorder, with an estimated incidence of ~1/20,000 live births in individuals of European ancestry, but it has an apparently lower incidence in most other ethnic groups (Opitz 1994; Cunniff et al. 1997; Kelley 1997). Patients typically show reduced levels of cholesterol and increased levels of 7-dehydrocholesterol (7DHC) and 8-dehydrocholesterol (8DHC) in plasma and tissues (Irons et al. 1993; Kelley 1995), which are caused by an apparent defect in the final step of cholesterol biosynthesis. This step is catalyzed by the enzyme Δ7-sterol reductase (DHCR7) (Tint et al. 1994), which is localized in the membrane of the endoplasmic reticulum (ER). SLOS is the best-studied example of genetically determined disorders of postsqualene cholesterol synthesis. Defects in an enzyme located upstream (Δ8–Δ9-sterol isomerase) cause X-linked Conradi Hünermann–type chondrodysplasia punctata (MIM 302960) (Braverman et al. 1999; Derry et al. 1999; Kelley et al. 1999). Before the discovery of the biochemical defect, two clinical forms of SLOS—known as "mild" (type I) and "severe" (type II)—had been distinguished clinically (Curry et al. 1987). However, the finding that both forms are associated with qualitatively the same abnormality of sterol metabolism indicated that the two forms represent two extremes of a single disorder (Cunniff et al. 1997).

We have recently cloned the cDNA for human DHCR7 (E.C.1.3.1.21), which catalyzes the conversion of 7DHC to cholesterol (Moebius et al. 1998), and have demonstrated that mutations in the gene cause SLOS (Fitzky et al. 1998). Mutations in *DHCR7* in patients

who have SLOS were also found by Wassif et al. (1998) and by Waterham et al. (1998). The gene, which was assigned to chromosome 11q13, contains nine exons and eight introns and spans ~14 kb (Fitzky et al. 1998). The first two exons are noncoding, whereas the start codon is localized in exon 3. The cDNA spans 2,646 bp and encodes a 475-amino-acid open reading frame (Moebius et al. 1998).

In the present study, PCR conditions were established to amplify all exons and exon-flanking intron regions of the *DHCR7* gene. SSCP, denaturing gradient-gel electrophoresis (DGGE), and automated sequencing of the PCR products were used to screen for mutations of *DHCR7* in 84 patients with biochemically confirmed SLOS, thereby permitting the investigation of phenotype-genotype correlation in SLOS.

Patients and Methods

Patients

The study population included 84 unrelated patients with SLOS who are from the United States, Germany, and the United Kingdom (table 1). All but one patient (D61, who had mostly American Cherokee heritage) were white. Of these 84 patients, 13 had been included in a previous analysis (Fitzky et al. 1998). In 62 patients, the diagnosis was established by quantification of 7DHC, by use of gas chromatography/mass spectrometry (Kelley 1995). When adequate clinical information was available, patients ($n = 55$) were further characterized by a scoring system, wherein malformations in each of 10 embryologically distinct areas were scored as either "1" or "2" and the sum normalized to 100 (Kratz and Kelley 1999). When patients whose SLOS was scored in this manner were adjudged qualitatively, by clinical geneticists, as having mild, moderate, or severe forms, the mean severity scores were 10, 39, and 72, respectively.

Mutation Analysis

Amplification.—High-molecular-weight DNA samples from the probands were isolated from either peripheral blood leukocytes or fibroblasts, by use of established procedures. Exons 1–9 and their flanking sequences were amplified by use of PCR (Saiki et al. 1985). The amplification primers for exons 3–9 were described elsewhere (Fitzky et al. 1998). In the present analysis, the 3′ and 5′ parts of the coding sequence of exon 9 were amplified separately. The primers used for exons 1 and 2 were as follows: 1 forward, gtccggccgctgccgaatggc; 1 reverse, cactgcgcacaccttcccctg; 2 forward, ggccttgagcgtctgccctctcc; and 2 reverse, catagcctgcgcccacgatccagg. The amplification was done over 35 cycles, with each cycle consisting of denaturation for 45 s at 95°C ; annealing for 45 s at 55°C (for exon 7), 57°C (for exons 1–3, 5, 6, and 8), and 62°C (for exons 4 and 9); and extension for 45 s at 72°C. The reaction mixture contained 50–100 ng DNA; 200 ng each primer; 200 μM each of dGTP, dCTP, and dATP; 800 μM dUTP; 3.5 mM $MgCl_2$; 10 mM Tris-HCl pH 8.8; and 1 U *Taq* polymerase (Dynazyme II; Finnzymes).

SSCP.—Exons 1–8 and the 5′ and 3′ ends of exon 9 were screened for mutations, by use of SSCP. A total of 7 μl PCR product was subjected to electrophoresis done on a mutation-detection–enhancement (MDE; FMC Bioproducts) gel and was then silver-stained as described elsewhere (Budowle et al. 1991).

DGGE.—For DGGE analysis of exons 4 and 9 (3′ end), 30-bp-long CG clamps were included at the 5′ end of the forward primer (exon 4) and at the 3′ end of the reverse primer (exon 9), respectively (Sheffield et al. 1989). The melting profiles of the fragments were analyzed by use of the computer algorithm Melt 87 (Lerman and Silverstein 1987). Gels were stained with ethidium bromide.

Sequencing.—The amplified DNA was subjected to electrophoresis on agarose gels from which the fragments were excised, were purified with QIA Quick PCR Purification Kit (Quiagen), and were sequenced, on the ABI Genetic Analyzer 310, with the Big Dye Terminator Cycle Sequencing Ready Reaction Kit (PE Biosystems).

In vitro mutagenesis, heterologous expression, and quantitative analysis of overexpressed protein.—Most of the identified point mutations were introduced, by in vitro mutagenesis, into the cDNA of human *DHCR7* fused with an N-terminal c-myc epitope, as described elsewhere (Fitzky et al. 1998). Wild-type and mutated cDNAs were subcloned into pCIneo (Promega) with *Xho*I-*Not*I restriction sites and were heterologously expressed in the HEK293-derived cell line tsA-201. For quantitative analysis of DHCR7 protein expression, 40 μg microsomal protein were separated on 12% (wt/vol) SDS-polyacrylamide gels, were transferred to polyvinyldifluoride membranes, and were immunostained, against the c-myc epitope, with 50 ng antibody 9E10 per ml (Oncogene Science). 9E10 c-myc immunoreactivity was visualized with the use of 35 μCi purified [^{125}J]-labeled anti-mouse immunoglobulin G (NEN) per ml and was quantified by membrane slicing and γ-counting (Fitzky et al. 1998). The data shown in table 2 are the results from three independent transfections.

Statistics

All patients were included in the genotype-phenotype analysis. To include patients whose exact severity scores were unknown but whose phenotype descriptions were available ($n = 14$), we assigned the scores "10, " for the

Table 1

Severity Scores, Age at Diagnosis, and Plasma Sterol Concentrations of Patients with SLOS

Patient (ID Number)	Severity Score	Age at Diagnosis (years)	Cholesterol (μg/ml)	8DHC (μg/ml)	7DHC (μg/ml)
Those with complete data set:					
1 (SLO1)	25	1.6	ND	45.7	156
2 (SLO3)	5	.6	1,160	69	117
3 (SLO7)	29	ND	342	94	107
4 (SLO8)	20	1.0	950	72	89
5 (SLO10)	25	.7	ND	85.6	162
6 (SLO11)	26	.11	ND	82	112
7 (SLO13)	10	.25	550	21	127
8 (SLO14)	Severe	.04	ND	ND	ND
9 (25644)	Severe	.015	162	169	173
10 (25719)	Severe	Fetus	ND_a	ND_a	ND_a
11 (D61)	17	3.4	1,059	26	26
12 (D64)	22	.01	145	45	52
13 (D65)	17	.5	543	95	177
14 (D66)	31	7.0	542	76	73
15 (D67)	12	27.0	833	84	110
16 (D68)	22	.1	310	73	88
17 (D69)	40	Fetus	ND	ND	ND
18 (D70)	Mild	Adult	1,190	ND	14
19 (D72)	69	.01	231	ND	218
20 (D73)	56	4.4	280	131	254
21 (D75)	19	.6	119	108	73
22 (D76)	39	.5	351	ND	470
23 (D78)	12	.03	1,000	58	41
24 (D79)	56	.01	ND	ND	ND
25 (D80)	100	Fetus	23.3	2.2	9.3
26 (D81)	28	1.42	42	68	148
27 (D82)	56	ND	ND	ND	6.9
28 (D85)	36	.25	236	109	231
29 (D86)	6	13.0	1,151	44	46
30 (D87)	75	Fetus	ND	ND	ND
31 (D89)	72	Amnion	18.9	ND	13.8
32 (D90)	50	.02	228	118	200
33 (D91)	17	7.0	510	62	59
34 (D92)	6	3.0	1,142	23	12
35 (D95)	39	.04	88	72	112
36 (D97)	6	.02	563	82	84
37 (D99)	Mild	35.0	1,272	259	134
38 (D100)	39	27.0	599	60	70
39 (D104)	Moderate	8.0	164	260	288
40 (D105)	75	.02	153	ND	138
41 (D106)	Severe	ND	170	ND	201
42 (D107)	56	.15	236	180	156
43 (D108)	Severe	Fetus	ND	ND	ND
44 (D111)	33	6.2	340	107	292
45 (D112)	33	21.0	500	101	221
46 (D113)	Severe	.17	98	72	96
47 (D114)	Moderate	10.0	373	108	108
48 (D115)	44	1.39	139	170	313
49 (D116)	22	8.0	748	68	70
50 (D117)	55	1.3	275	50	105
51 (D118)	Mild	10.0	1,149	23	14
52 (D119)	17	.1	326	17	19
53 (D120)	22	.73	315	46	140
54 (D121)	50	.04	40	46	29
55 (D123)	33	.01	170	160	170
56 (D124)	25	.03	41	43	104

(*continued*)

Table 1 Continued

Patient (ID Number)	Severity Score	Age at Diagnosis (years)	Cholesterol (μg/ml)	8DHC (μg/ml)	7DHC (μg/ml)
57 (D125)	11	41.0	1,207	122	291
58 (D127)	33	26.58	912	169	371
59 (D128)	39	.83	69	92	217
60 (D129)	13	5.00	1,385	61	84
61 (D130)	38	.91	621	73	143
62 (D138)	31	.01	410	75	85
63 (D139)	6	5.75	1,377	10	8
64 (D141)	Moderate	3.8	432	94	111
65 (D142)	Severe	Fetus	ND	ND	ND
66 (D144)	38	16.33	410	46	60
67 (D145)	6	3.8	1,738	18	18
68 (D152)	Moderate	10.75	1,358	29	62
69 (D163)	>75	Fetus	30[b]	5	20
Those for whom no severity score was available:					
70 (SLO2)	ND	2.5	910	ND	432
71 (SLO4)	ND	ND	1,550	ND	100
72 (SLO5)	ND[c]	.06	340	ND	67
73 (SLO6)	ND	1	1,152	77.5	100
74 (SLO9)	ND	.5	700	ND	89
75 (SLO12)	ND[c]	.08	340	140	357
76 (1980)	ND	ND	ND	ND	ND
77 (8a)	ND	ND	ND	ND	ND
78 (860)	ND	ND	280	ND	438
79 (13141)	ND	7	3.51	420	378
80 (23212)	ND	.02	81	317	469
81 (25718)	ND[c]	.104	313	84.6	157
82 (D84)	ND	5.0	906	37	39
83 (D122)	ND	ND	645	92	220
84 (D135)	ND	ND	ND	ND	ND

NOTE.—ND = not determined.
[a] Elevated 8DHC in amniotic fluid and fetal tissue.
[b] Amniotic fluid.
[c] Most likely severe, because of very early death.

mild phenotype; "39, " for the moderate (classic) phenotype; and "72," for the severe phenotype, as noted earlier. The Kruskal-Wallis, Mann-Whitney, and χ^2 goodness-of-fit tests were applied as indicated. Spearman correlation coefficients (R_S) and linear regression were calculated by use of the software package SPSS for Windows (release 8.0.0. 1997; SPSS). Analysis of variance (ANOVA) was used to compare the variance of quantitative biochemical variables both within and between genotypes.

Results

Mutational Spectrum

All of the exons encoding the open reading frame, as well as the untranslated exons 1 and 2, were screened by means of either SSCP (exons 1–9 [5′ end] and 9 [3′ end]) or DGGE (exons 4 and 9 [3′ end]) performed in the 84 patients. Exons showing shifted or ambiguous banding patterns were sequenced. However, in those patients for whom either no mutation or only one mutation was found by use of this strategy, all exons were sequenced. This resulted in a mutational detection rate of 96%. In all patients with biochemically confirmed SLOS, we found ⩾1 mutation. For only six patients were we unable to identify a 2d mutation. Three mutations were found in two of the patients (D80 and D111). This was confirmed by analysis of the parents. The genotypes of these patients were IVS 8–1G→C; R 352Q/R352Q (D80) and IVS 8-1G→C; T93 M/IVS 8-1G→C (D111).

We identified a total of 40 different mutations, several of which were recurrent, in the patients. In addition, several polymorphic silent mutations, some of which have previously been described elsewhere (Fitzky et al. 1998), were found. Newly identified sequence variants causing no amino acid change were 285A→G, 969G→T, 1350C→G, and −223T→C. Mutation −223T→C occurred in 2/40 healthy control individuals.

Table 2 lists all identified SLOS mutations. The majority (36/40 [90%]) were missense mutations. The

Table 2

Spectrum of SLOS-Causing Mutations in the *DHCR7* Gene

Mutation Class and Nucleotide Change	Exon	Effect on Coding Sequence	Conserved Amino Acid	Localization in the Protein	Expression (%)
Wild type:					
None					100
0:					
IVS8-1G→C	9	Frameshift			
(385–412 and IVS5+1-5)del	5	Frameshift			
720–735 del	6	Frameshift			
453G→A	6	W151X	No		0
TM:					
151C→T	4	P51S	Yes	TM	
278C→T	4	T93M	Yes	TM	<5
296T→C	4	L99P	Yes	TM	<5
321G→C	4	Q107H	Yes	TM	
326T→C	5	L109P	Yes	TM	
440G→A	6	G147D	Yes	TM	
461C→T	6	T154M	Yes	TM	<5
470T→C	6	L157P	No	TM	
506G→C	6	S169L	No	TM	
549G→C	6	W182C	Yes	TM	<5
724C→T	7	R242C	Yes	TM	<3
740C→T	7	A247V	Yes	TM	5
841G→A	8	V281M	Yes	TM	
866C→T	8	T289I	Yes	TM	
931T→G	8	C311G	Yes	TM	
932G→A	8	C311Y	Yes	TM	
970T→C	9	Y324H	Yes	TM	
976C→T	9	V326L	Yes	TM	<10
1055G→A	9	R352Q	Yes	TM	
1054C→T	9	R352W	Yes	TM	<3
1058T→C	9	V353A	Yes	TM	
1222T→C	9	Y408H	Yes	4th Cytoplasmic loop/TM	
1228G→A	9	G410S	Yes	4th Cytoplasmic loop/TM	4
1228G→C	9	G410R	Yes	4th Cytoplasmic loop/TM	
4L:					
1084C→T	9	R362C	Yes	4th Cytoplasmic loop	
1139G→A	9	C380Y	No	4th Cytoplasmic loop	40
1138T→C	9	C380R	No	4th Cytoplasmic loop	5
1138T→A	9	C380S	No	4th Cytoplasmic loop	
1190C→T	9	S397L	Yes	4th Cytoplasmic loop	
1210C→T	9	R404C	Yes	4th Cytoplasmic loop	2
1210C→A	9	R404S	Yes	4th Cytoplasmic loop	
CT:					
1327C→T	9	R443C	Yes	C-terminus	<3
1337G→A	9	R446Q	Yes	C-terminus	
1342G→C	9	E448Q	Yes	C-terminus	
1342G→A	9	E448K	Yes	C-terminus	5
1349G→T	9	R450L	Yes	C-terminus	90

four nonmissense mutations included one out-of-frame 15-bp deletion (720–735 del), a 33-bp deletion comprising 5 bp of a splice-donor site (385–412del and IVS 5+1–5del), one splice-site mutation (IVS8-1G→C) resulting in a 134-bp insertion because of alternative splicing, and a nonsense mutation (W151X); all of these nonmissense mutations are predicted to result in truncated proteins and may also cause mRNA instability (Maquat 1996). Mutations occurred in the translated exons 4–9 but were clustered in exon 9, in which ~50% (21/40) of the mutations were found. Several codons had two or three different mutations (e.g., C380S, C380R, and C380Y) (fig. 1).

The highest allele frequency was observed for the splice-site mutation IVS8-1G→C, which was found 48 times among the 168 SLOS alleles ($P = .29$ of all SLOS alleles). Four homozygotes for this mutation were found, which is close to the expected number (table 3). The

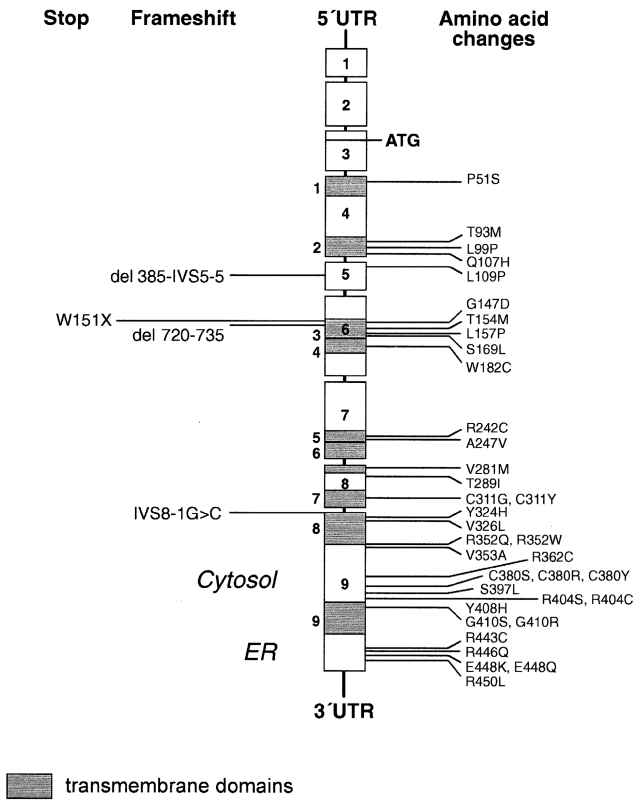

Figure 1 Distribution of SLOS-causing mutations in the *DHCR7* gene. Exons are drawn to scale; introns are not drawn to scale. The ATG initiation codon is indicated. The hatched boxes with roman numerals denote the transmembrane domains. The cytosolic loop in exon 9 (cytosol) and the C-terminus, assumed to be located in the lumen of the ER, are indicated.

missense mutation R404C also occurred with a frequency of SLOS alleles that was >.1, and mutations T93M, W151X, and V326L had frequencies >.05. It is notable that two of the truncating mutations (IVS8–1G→C and W151X) were among the five most frequent SLOS mutations identified, accounting for one-third of all SLOS mutations.

Homozygotes were also identified for R404C, W151X, R352Q, and C380Y. None was from a known consanguineous marriage. However, for one of the mutations (R404C), significantly more homozygotes (n = 5) were identified than were expected (n = 1). Three of the five R404C homozygotes were from the same Louisiana parish and have in common an ancestor of French origin dating back to the early 18th century. Hence, this mutation most likely represents a French founder mutation.

Of the 36 missense mutations, 31 affected conserved residues (i.e., residues identical in the human, mouse, and plant *DHCR7* genes [Fitzky et al. 1998]). Only five mutations changed a nonconserved residue. Three of these changed the same Cys residue (C380 to Y, R, and S, respectively), and one introduced a proline into a presumably α-helical hydrophobic transmembrane domain (L157P). All of these mutations, by virtue of the type of amino acid change introduced, are likely to be SLOS-causing mutations.

The parents of patients D75, SLO14, and 25720, who, according to the results of SSCP/sequencing, appeared to be homozygotes, were analyzed, to establish that they were true homozygotes and that they were not, for example, carrying a deletion for one allele. In all cases, both parents were heterozygous for the respective mutation. Together with the high mutation-detection rate (96%), this result suggests that no frequent large deletions or other SLOS-causing mutations exist and that patients identified as homozygotes are true homozygotes.

Localization of Missense Mutations in the Protein and Classification of Mutations

The DHCR7 protein is localized in the membrane of the ER and has nine putative transmembrane domains and one large cytoplasmic loop (the 4th cytoplasmic loop) (Fitzky et al. 1998; Moebius et al. 1998). The C-terminus is predicted to be located in the lumen of the ER. The 36 missense mutations have a characteristic distribution along this topography (fig. 1). Twenty-one mutations are located in the transmembrane domains, three are at the border of transmembrane domains 8 and

Table 3

Frequent Mutations in the *DHCR7* Gene of 84 Patients with SLOS

Mutation[a]	Exon	No. of Alleles (n = 168)	Frequency (P)	No. of Homozygotes	
				Identified	Expected
IVS8-1G→C	9	49	.29	4	7.0
R404C	9	18	.11	5	1.0
T93M	4	14	.08	...	.6
W151X	6	14	.08	1	.6
V326L	9	12	.07	...	.43
R352W	9	5	.03	...	.11
S169L	6	4	.02	...	.03
E448K	9	3	.02	...	.03
R242C	7	3	.02	...	.03
Y324H	9	3	.02	...	.03
R352Q	9	3	.02	1	.03
P51S	4	2	.01	...	.01
L99P	4	2	.01	...	.01
T154M	6	2	.01	...	.01
T289I	8	2	.01	...	.01
C380Y	9	2	.01	1	.01
R404S	9	2	.01	...	.01
G410S	9	2	.01	...	.01
R446Q	9	2	.01	...	.01

[a] All mutations other than those listed were represented by a single chromosome.

9, seven are in the 4th cytoplasmic loop, and five are located in the C-terminus of the protein. Hence, there is clustering of the missense mutations in the transmembrane domains. These mutations are likely to affect protein stability, which has already been demonstrated for some of the mutations (Fitzky et al. 1998). Notably, all but one (R450L) of the missense mutations analyzed in the present study also reduced protein expression (table 2). At least some of the mutations in the C-terminal ER domain of *DHCR7*, including R450L, also reduce the enzymatic activity (B. U. Fitzky, unpublished data). On the basis of the localization of the amino-acid sequence changes in the protein and their presumed functional consequences, we categorized SLOS mutations into four classes (table 2): truncating mutations predicted to result in functional null alleles (0 mutations); mutations located in or close to the transmembrane domain (TM mutations); mutations in the 4th cytoplasmic loop (4L mutations); and mutations affecting the C-terminal domain (CT mutations).

Genotype-Phenotype Correlation

The classification of mutations into four distinct types (O, TM, 4L, and CT mutations) was used to analyze whether a relation exists between *DHCR7* genotypes and SLOS phenotypes. Of the 10 possible genotype combinations, 7 (0/0 = 7, TM/TM = 6, 4L/4L = 6, 0/TM = 40, 0/4L = 10, TM/4L = 2, and TM/CT = 7) were represented in the sample.

Genotypes were related to the biochemical phenotypes, by comparison of various sterol metabolite concentrations and their ratios with the clinical phenotypes and by application of the described scoring system. When total cholesterol, 7DHC, 8DHC, the 7DHC/cholesterol ratio, and the sum of 7DHC plus 8DHC expressed as a fraction of total sterols (DHC fraction) were compared both within and between genotypic classes, they were more similar within genotypes than between genotypes (ANOVA, $P = .001$). We first analyzed the relation between the patients' sterol parameters and their severity scores, and we found a significant correlation between the DHC fraction and the severity score (fig. 2*A*). There was also a clear correlation between the DHC fraction and the genotypes ($R_s = 0.548$, $P < .001$) (fig. 2*B*). Patients with two TM mutations or with one TM and one CT mutation had the lowest DHC fraction, on average, whereas those with two 4L mutations, those who were homozygotes and compounds for 0 mutations, and those with one 0 and one 4L mutation had the highest DHC fractions. Patients with 0/TM and 4/TM mutations had intermediate values. Despite the substantial differences among the various means, there was broad overlap between the groups. The correlation of genotypes with the sterol levels suggested that the geno-

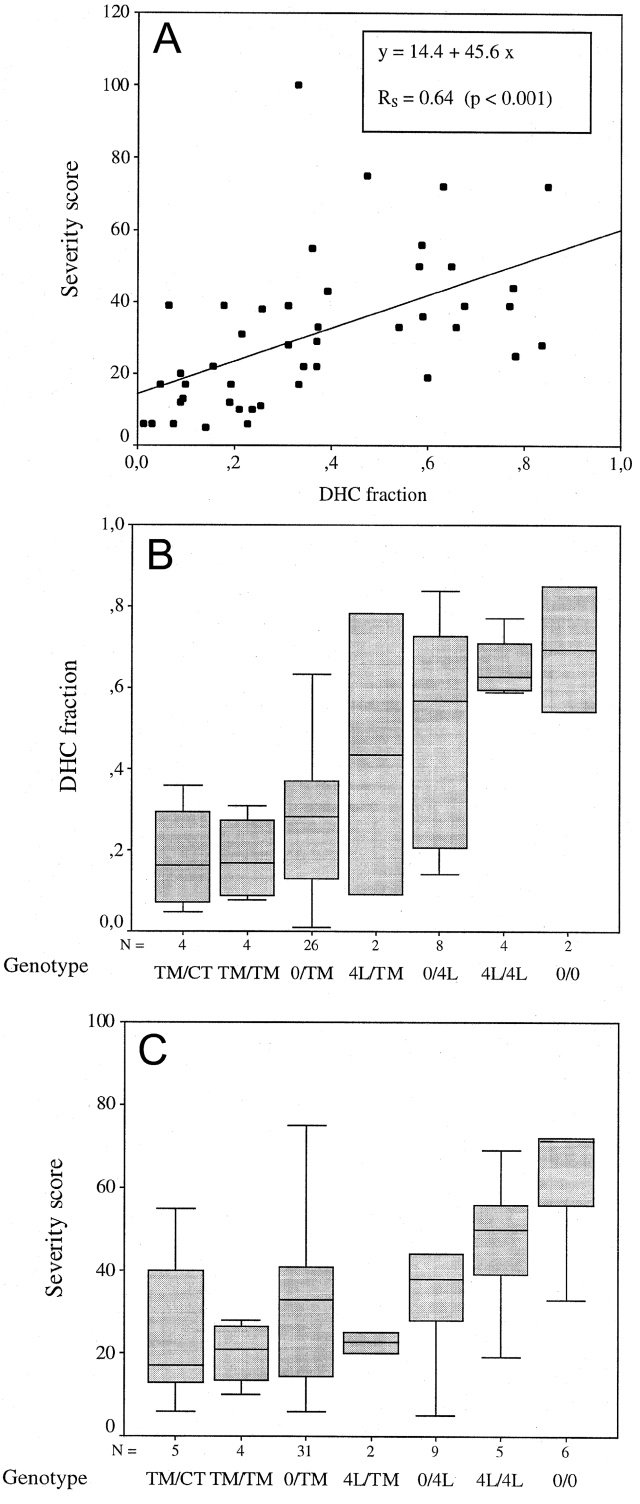

Figure 2 *A*, Correlation between severity score and DHC fraction. R_s and linear regression coefficients are shown. *B*, Box-plot diagram illustrating the distribution of DHC fraction values within genotypes. *C*, Box-plot diagram illustrating the distribution of severity scores within genotypes. Black lines within boxes in *B* and *C* represent medians. Boxes represent the range, accommodating 95% of values, and vertical lines represent the total range for the group.

types might also correlate with the severity scores. Indeed, average disease severity increased in almost the same order with genotypes as did genotypes with the DHC fraction. Patients in the TM/TM and TM/CT groups were, on average, less severely affected than were patients with two 0 mutations or those with two mutations in the 4th cytoplasmic domain (fig. 2*C*). For this correlation, the coefficient was $R_s = .370$ ($P < .006$). Thus, in summary, we found, among our patients with SLOS, a strong correlation of both the genotypes and the cholesterol metabolites with their clinical severity. We further evaluated the genotype distribution among patients in the three classes of severity: severe (score >50), moderate (score >25 and <50), and mild (score <25). Genotypes 0/0 and 4L/4L were significantly more frequent in patients with a severe phenotype, whereas genotypes TM/CT and TM/TM occurred predominantly in patients with a mild/moderate phenotype. This difference was significant ($P = .001$).

Correlation of Genotypes with Age at Diagnosis

We compared the age at diagnosis of term-delivered patients for the three groups of severity. The group with the lowest severity score (<25) had a significantly higher age at diagnosis (mean age = 8.6 years ± 12.4; $n = 20$), whereas the group with the highest severity score (>50) included the youngest patients (mean .29 years ± .50; $n = 10$). The difference in age at diagnosis between groups was significant (Kruskal-Wallis test $P = .050$). Because age at diagnosis correlates with clinical severity, it should also correlate with genotype. The genotype distributions were therefore compared between patients in whom SLOS was diagnosed either at age <1 year ($n_1 = 37$) or later in life ($n_2 = 29$), by means of the Mann-Whitney test, and they were found to be significantly different (table 4).

SLOS Phenotypes in Homozygotes or Compounds for Presumed Functional Null Alleles of Δ7-Sterol Reductase and Mutations in the Most Severe Cases

As shown in the genotype-phenotype correlation studies, patients with two functional null *DHCR7* alleles have the most severe phenotypes. Of the four patients homozygous for the IVS8-1G→C mutation, three were severely affected and were detected by prenatal ultrasound. These pregnancies were terminated. The other IVS8-1G→C homozygote was moderately affected. The only patient homozygous for the W151X stop mutation also had a severe phenotype detected prenatally. The compound heterozygote for W151X and IVS8-1G→C had a severe phenotype (severity score 56) and died at age 2 d.

In all, we studied 16 cases with a severity score >50 (table 5). The frequency of 0 alleles (.59) and of null and 4L alleles combined (.78) was significantly higher than that in the mild/moderate group ($P = .05$), and a significant number of patients (10/16) were 0 and/or 4L homozygotes or compounds. Only one had neither a 0 mutation nor a class 4L mutation.

Discussion

We analyzed the genotypes of a large cohort of patients with biochemically and clinically characterized SLOS. This allowed us to address how the genotype determines the biochemical and clinical phenotype. Two groups of patients are from England and Germany, respectively, but the majority of the patients are Americans of European descent. Our sample therefore represents major European populations. The large number of patients analyzed makes it likely that our study covers most of the existing spectrum of frequent mutations in the *DHCR7* gene.

In 168 alleles, we found a total of 40 different mutations, of which 36 were missense mutations. The ma-

Table 4

Distribution of *DHCR7* Genotype Classes, in Relation to Age at Diagnosis and Severity, in Patients with SLOS

	Genotype Class						
Characteristic	TM/TM	TM/CT	0/TM	4L/TM	0/4L	4L/4L	0/0
Age (years):							
≤1	4	2	16	2	6	4	6
>1	2	4	19	0	4	1	1
Disease severity:							
Mild[a]	2	3	12	1	2	1	0
Moderate[b]	2	1	13	1	5	2	1
Severe[c]	0	1	5	0	2	2	6

[a] Severity score <25.
[b] Severity score 25–50.
[c] Severity score >50.

Table 5

Mutations in the *DHCR7* Gene in Patients with SLOS Who Have a Severity Score >50

Patient	Severity Score	Genotype	Mutation Class	Remarks
SLO14	Severe	IVS8-1G→C/IVS8-1G→C	0/0	Died at age 22 d
D79	56	IVS8-1G→C/W151X	0/0	Died at age 2 d
D89	72	W151X/W151X	0/0	Termination
D108	Severe	IVS8-1G→C/IVS8-1G→C	0/0	Termination
D163	>75	IVS8-1G→C/IVS8-1G→C	0/0	Termination
25719	Severe	IVS8-1G→C/IVS8-1G→C	0/0	Termination
D72	69	R404C/R404C	4L/4L	Died at age 6 wk
D107	56	R404C/R404C	4L/4L	Diagnosed at age .15 years, died at age 1 year
D105	75	W151X/S397L	0/4L	Diagnosed at age .02 years, died during first weeks of life
25644	Severe	IVS8-1G→C/ R404C	0/4L	Baby, severe SLOS
D73	56	IVS8-1G→C/L109P	0/TM	Diagnosed at age 4.4 years
D142	Severe	IVS8-1G→C/T93M	0/TM	Termination
D80	100	IVS8-1G→C/R352Q	0/TM	Termination
D87	75	IVS8-1G→C/V326L	0/TM	Termination
D113	Severe	W151X/R352Q	0/TM	Diagnosed at age .17 years
D117	55	V281M/R446Q	TM/CT	Diagnosed at age 1.3 years

jority of mutations was found in exons 9 (111), 6 (24), and 4 (19), so that analysis of these three exons will identify >90% of the mutations. Of the 40 mutations, only 4 truncate the protein and remove essential functional domains. These four mutations (W151X, IVS8-1G→C, 385-412del+IVS5+1-5del, and 720-735del) presumably result in functional *DHCR7* null alleles. In fact, on western blotting, no protein was present in HEK cells transfected with the W151X cDNA (table 2), and there also was no enzyme activity present (B. U. Fitzky, unpublished data).

Given that only a few null alleles exist, it is surprising that two of them (IVS8-1G→C and W151X) accounted for more than one-third of all mutations. Why are 0 mutations that frequent? To explain this conundrum, we have to consider two possibilities. The two 0 mutations (IVS8-1G→C and W151X) could confer a significant heterozygote advantage. If the 0 mutations are indeed selected, it remains to be clarified why null alleles resulting in 50% of wild-type activity in heterozygotes should be preferred over hypomorphic alleles resulting in, for example, 55% of biochemical activity in heterozygous carriers. In contrast, the high frequency of 0 mutations could simply reflect the expansion of ancient populations carrying these mutations. This view is supported by the finding that two alleles carrying two different SLOS-causing mutations were identified, both of which involved the frequent IVS8-1G→C mutation. Hence, this mutation seems to be old. Approximately another one-third of the SLOS chromosomes contain four frequent missense mutations (R404C, T93M, V326L, and R352W) in the *DHCR7* gene. Even so, it seems conceivable that one or two mutations (e.g., IVS8-1G→C) were preexistent in an expanding population. It is intriguing that the six mutations that account for two-thirds of all *DHCR7* alleles should have existed in the people that populated Europe. SLOS is virtually absent in Africans and Asians. Haplotype analysis has to be applied to establish the history and spatial distribution of frequent *DHCR7* mutations and to discern both founder effects from recurrent mutations and possible heterozygote advantage. In the absence of the six most frequent mutations, the SLOS would be a rare disorder in Europeans.

Most of the missense mutations target residues that are conserved between plants and vertebrate enzymes, suggesting that, in the protein, these sites are critical for function (table 2). Of 30 mutated amino acid residues, 6 are hit two or even three times (table 2), underscoring their potential importance for catalysis or protein folding. Two-thirds of the missense mutations were localized either within or adjacent to the proposed nine transmembrane segments (table 2 and table 3). Such clustering in transmembrane domains is known from disease-causing mutations in other genes (e.g., PMP22) (Nelis et al. 1999). Whereas no mutations were found in the N-terminus, several mutations were localized in the C-terminus. One-fourth of the mutations targeted the 4th cytoplasmic loop between transmembrane segments 8 and 9. This region was suggested to bind NADPH, the indispensable cofactor of 7DHC reduction (Fitzky et al. 1999). Even though a putative sterol-binding site was identified in TMS5-8 (Bae et al. 1999), no functional domains of the enzyme have been confirmed experimentally. Therefore, in the present study, we have arbitrarily classified the missense mutations, according to their topological localization in the transmembrane region (TM), the 4th cytoplasmic loop (4L), and the C-terminus (CT). Functional analysis, by expression of *DHCR7* mutations in mammalian cells, demonstrated

that all but one of the missense mutations result in unstable protein (table 2). The only mutation that has normal protein expression (R450L) was present in patient D139, who has one TM mutation and one CT mutation (genotype R242C, R450L). This is particularly interesting, because this mildly affected patient has biochemical characteristics that differ significantly from those of other patients with SLOS (Anderson et al. 1998). Further biochemical studies are needed to characterize this and other CT mutations in detail.

A characteristic feature of SLOS is the phenotypic variability of a few severely affected patients (n = 16 in this study) and a larger number of patients with a mild or moderate clinical phenotype (n = 49 in this study). We sought to address whether the clinical and biochemical phenotypes are associated with certain genotypes and found a strong correlation between clinical and biochemical phenotype and both the type and localization of mutations. Mild phenotypes were more frequent among patients with two missense mutations in either the transmembrane segments or the C-terminus (TM/TM, TM/CT), whereas severe phenotypes were more abundant among patients who are homozygous or compounds for 0 mutations or missense mutations in the 4th cytoplasmic loop (0/0, 0/4L, and 4L/4L). Therefore, not only null alleles but, also, mutations in the 4th cytoplasmic loop of the enzyme are a major determinant of phenotypic severity. It would be highly desirable to identify the function of this domain.

Although our study unequivocally established that the clinical and biochemical phenotypes correlate with the genotype, we cannot predict the phenotype from the genotype, and vice versa, probably because the correlation is obscured by two factors: first, additional environmental or genetic determinants that remain to be identified can modify the phenotype; second, our classification of mutations does not reflect the unknown biochemical consequences of all mutations within a given class in vivo. Identification of those frequent patients who have a 0 mutation combined with a missense mutation (0/TM, 0/4L) paves the way for analysis of the functional consequences of missense mutations in vivo, to further refine our classification of mutations. A side-by-side comparison of environmental factors, metabolic characteristics, and genetic markers in patients with identical genotypes might identify the postulated modifiers of the phenotype. Consideration of the genotype may also be important in studies that measure the response of patients to cholesterol feeding, which reportedly has beneficial effects on the behavioral phenotype of patients with SLOS (Irons et al. 1994; Nwokoro and Mulvihill 1997; Cunniff et al. 1997; Angle et al. 1998). The response of patients to dietary cholesterol may also depend on genotype.

An indispensable step in the biosynthesis of cholesterol from the intermediate lanosterol is the reduction of the D7-bond of 7DHC. In the absence of the enzyme D7-sterol reductase because of two *DHCR7* null alleles, the reduction of 7DHC should be abolished. A fundamental biological question is whether vertebrates can develop without endogenous cholesterol biosynthesis. Patients with two 0 mutations might be a genetic model for a complete block in cholesterol synthesis. Seven patients with two 0 mutations indeed had a severe phenotype that led to termination of the pregnancy in four patients and early postnatal death in three (table 5). However, one patient had a moderate phenotype despite two 0 mutations (IVS8-1G→C homozygote), implying that other, yet-to-be identified determinants of the phenotype may exist. There are at least four possibilities to explain the presence of cholesterol and a moderate phenotype in living patients with SLOS with two 0 mutations: (i) there might be another source of *DHCR7* activity, (ii) there could be another pathway of cholesterol synthesis not requiring *DHCR7,* (iii) the IVS8-1G→C splice mutation may be leaky, allowing for the presence of some normal mRNA, and (iv) there may be exogenous sources of cholesterol (e.g., transport of lipoproteins through the placenta).

The detailed mechanism(s) by which mutations in the *DHCR7* affect cholesterol metabolism and result in the SLOS phenotype still need to be clarified. Ultimately, this will help in the understanding of how cholesterol and the intermediates of sterol metabolism determine morphogenesis and brain function.

Note added in proof.—After submission of the manuscript, seven *DHCR7* mutations were detected in 12 additional patients with SLOS (R. Berberich and H. J. Menzel, personal communication).

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Electronic-Database Information

Accession numbers and URLs for data in this article are as follows:

Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.nih.gov/Omim/ (for SLOS [MIM 270400] and X-linked chondrodysplasia punctata–type Conradi Hünermann [MIM 302960])

SPSS, http://www.spss.com/ (for SPSS for Windows software)

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