ORIGINAL ARTICLE

Abnormal sterol metabolism in holoprosencephaly: studies in cultured lymphoblasts

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Background: Holoprosencephaly (HPE) is the most common structural malformation of the developing forebrain in humans. The aetiology is heterogeneous and remains unexplained in approximately 75% of patients.

Objective: To examine cholesterol biosynthesis in lymphoblastoid cell lines of 228 patients with HPE, since perturbations of cholesterol homeostasis are an important model system to study HPE pathogenesis in animals.

Methods: An in vitro loading test that clearly identifies abnormal increase of C27 sterols in lymphoblast-derived cells was developed using [2-¹⁴C] acetate as substrate.

Results: 22 (9.6%) HPE cell lines had abnormal sterol pattern in the in vitro loading test. In one previously reported patient, Smith–Lemli–Opitz syndrome was diagnosed, whereas others also had clearly reduced cholesterol biosynthesis of uncertain cause. The mean (SD) cholesterol levels were 57% (15.3%) and 82% (4.7%) of total sterols in these cell lines and controls, respectively. The pattern of accumulating sterols was different from known defects of cholesterol biosynthesis. In six patients with abnormal lymphoblast cholesterol metabolism, additional mutations in genes known to be associated with HPE or chromosomal abnormalities were observed.

Conclusions: Impaired cholesterol biosynthesis may be a contributing factor in the cause of HPE and should be considered in the evaluation of causes of HPE, even if mutations in HPE-associated genes have already been found.

Holoprosencephaly (HPE) is a common congenital malformation characterised by incomplete cleavage of the embryonic forebrain.¹ HPE occurs in approximately 1 in 10 000 births and in 1 in 250 embryos,¹ and ranges from cyclopia with a proboscis and a single prosencephalic vesicle to obligate HPE carriers with a normal central nervous system by MRI and normal facial appearance. Multiple genetic causes of HPE have been identified, the most common being cytogenetic abnormalities.² Approximately 20% of infants with normal karyotypes have missense mutations in one of the four genes currently linked to isolated, sporadic and familial, non-syndromic HPE: Sonic Hedgehog (*SHH*),³ Zinc finger transcription factor 2 (*ZIC2*),⁴ Sine oculis homeobox (*Drosophila*) homologue 3 (*SIX3*),⁵ and TG-interacting factor (*TGIF*).⁶ Microdeletions in these genes occur in another 5%.⁷ However, the underlying cause of HPE remains unknown in most cases.

Among a variety of embryological toxins that can elicit abnormal craniofacial development including HPE,⁸ inhibitors and other perturbations of cholesterol biosynthesis have been shown to be important factors, most likely because cholesterol is required for *SHH* signal response.⁹ For example, HPE is induced by exposure of rat embryos to inhibitors of the distal steps of cholesterol biosynthesis (AY9944, BM15766 and triparanol; fig 1), causing accumulation of cholesterol precursors and decreased cholesterol concentrations.^{10–11} HPE-spectrum malformations also occur in about 5% of patients with Smith–Lemli–Opitz syndrome (SLOS), a defect in the last step of cholesterol biosynthesis catalysing the conversion of 7-dehydrocholesterol to cholesterol,¹² and in mouse embryos deficient in megalin, a member of the low-density lipoprotein receptor family that is expressed in embryonic neuroectoderm.¹³ Finally, certain plant alkaloids (cyclopamine, jervine) that inhibit intracellular cholesterol transport promote HPE in chick

and mouse embryos.¹⁴ Although these compounds were first thought to cause HPE by reducing *SHH* signalling, a study demonstrated that cyclopamine directly inhibits Smoothed, a transmembrane protein involved in embryonic signalling distal to *SHH*.¹⁵

Many studies have shown that the expressed HPE phenotype is extremely variable even in families segregating a heterozygous HPE-causing mutation. Preliminary evidence suggests that this phenotypic variability may reflect a combination of genetic, epigenetic and environmental influences,¹⁶ including anomalies in cholesterol metabolism.¹⁷ Since no systematic investigations of cholesterol metabolism in patients with HPE have been reported, we examined cholesterol biosynthesis in lymphoblastoid cell lines of a large cohort of patients with HPE with a newly developed, sensitive test system.

MATERIALS AND METHODS

Materials

[2-¹⁴C]acetic acid (56 mCi/mmol) was purchased from Hartmann Analytic, Braunschweig, Germany. Cholesterol, desmosterol, lathosterol, 7-dehydrocholesterol, lanosterol, 1,4-bis(2-chlorobenzylaminomethyl) cyclohexane dihydrochloride (AY9944), cabosil M-5, insulin-transferrin-sodium selenite media supplement and sodium acetate were purchased from Sigma Aldrich, Taufkirchen, Germany. 5 α -Cholestane was from Merck, Darmstadt, Germany. N-Methyl-N-trimethylsilylheptafluor(o)

Abbreviations: DHC, dehydrocholesterol; ER, endoplasmic reticulum; GC-MS, gas chromatography-mass spectrometry; HPE, holoprosencephaly; PBS, phosphate-buffered saline; LC, lymphoblastoid cell; RF, retention fraction; *SHH*, Sonic Hedgehog; SLOS, Smith–Lemli–Opitz syndrome; TLC, thin-layer chromatography

butyramide (MSHFBA) was from Macherey-Nagel, Düren, Germany.

Hexane, ethanol, benzene, ethylacetate, NaOH, Na₂CO₃ and KOH were purchased from Roth, Karlsruhe, Germany. Silica gel 250 μm AgNO₃ thin-layer chromatography (TLC) plates (10% silver nitrate) were from Analtech, Newark, Delaware, USA. Bromothymol blue was purchased from Alltech Grom, Rottenburg, Germany. Dulbecco's modified Eagle medium and phosphate-buffered saline (PBS) were obtained from Cell Concepts, Umkirch, Germany. RPMI 1640, MEM-NEAA (non-essential amino acids), fetal calf serum, amphotericin B,

penicillin/streptomycin were from PAA Laboratories, Cölbe, Germany. Gentamycin was obtained from Invitrogen, Karlsruhe, Germany and the mycoplasma test was from Cambrex BioScience, Verviers, Belgium.

Patients

Lymphoblastoid cell (LC) cultures from 214 patients with various forms of HPE, 13 parents and one sibling were included in this study. In all, 100 individuals were male, 125 female, and in 3 patients the gender was unknown because of early fetal death. All cell lines and submicroscopic deletions in four genes

Figure 2 Influence of different organic solvents on the sterol separation. 10 μ M of unmarked sterol standards (lanosterol, cholesterol, desmosterol, lathosterol and 7-dehydrocholesterol (DHC)) was applied on a 10% silver nitrate silica plate and resolved with different mobile phases (two developments each). (A) Benzene/ethylacetate 5/1 v/v, (B) benzene/ethylacetate 9/1 v/v, (C) benzene/ethylacetate 19/1 v/v, and (D) primary development: chloroform/acetone 27.6/1 v/v; RF, retention fraction; secondary development: diethyl ether/n-hexane 5/1 v/v.

known to be associated with HPE (*SHH*, *ZIC2*, *SIX3* and *TGIF*) were analysed for mutations. Control human LC lines were obtained from nine healthy individuals. Blood sampling and experiments in LC cultures were performed after informed consent was obtained. These studies were approved by the Institutional Review Board of the National Human Genome Research Institute, NIH-1195-1(8-98), and by the ethics committee of the medical faculty, University of Heidelberg (No. 211/2002), Germany.

Lymphoblastoid cell culture

For generation of LC lines, B-lymphocytes of 0.5–10 ml venous EDTA blood samples from patients with HPE and healthy volunteers were isolated using Ficoll density gradient centrifugation, immortalised by exposure to Epstein–Barr virus (EBV) supernatant and cultivated for at least 6 weeks before application.¹⁸

LCs were cultivated to saturation density at 37°C in RPMI 1640+L-glutamine supplemented with penicillin, streptomycin, amphotericin B, gentamycin and MEM-NEAA with 100 ml/l fetal calf serum. Each LC culture was tested for contamination by *Mycoplasma* species before the experiments.

In vitro [2-¹⁴C]acetate loading test

LCs were preincubated in lipid-depleted cell culture medium, prepared according to a published procedure,¹⁹ for 24 h to activate endogenous cholesterol synthesis. Briefly, lipid depletion was achieved by mixing fetal calf serum with Cabosil M5. The mixture was stirred overnight, centrifuged and the supernatant was filtered sterile. The cholesterol content of the lipid-depleted medium was 0.12 mg/dl. Cell viability was determined by trypan blue exclusion, and 4×10^6 viable cells were washed in PBS, incubated with lipid-depleted medium containing 2.5 μ Ci

[2-¹⁴C]acetate for 2 h and chased in lipid-depleted medium containing 144 mM sodium acetate for another 2 h. Cells were washed, harvested by centrifugation, and homogenised in 1000 μ l of a solution containing 200 mM sodium carbonate and 100 mM NaOH. The homogenate was stored at –80°C until extraction. 500 μ l of the sample was hydrolysed at 60°C for 30 min with 1 ml degassed 4% (weight/volume (w/v)) KOH in ethanol, followed by extraction with n-hexane. Neutral sterols were separated on a silver nitrate silica gel TLC plate with benzene/ethylacetate (9/1 v/v) as mobile phase. The plates were thoroughly dried and development in the same solvent mixture repeated. Quantification of sterols was achieved by phosphoimaging (phosphoimager FLA 3000, Fujifilm, Tokyo, Japan) and subsequent densitometric analysis.

Differentiation and quantification of sterols by gas chromatography-mass spectrometry

LCs were cultured in lipid-depleted medium for 72 h. A total of 4×10^6 viable cells were harvested by centrifugation, washed with PBS and hydrolysed with ethanolic KOH as described above. 5 α -Cholestane was added as internal standard. After extraction with water and n-hexane, the sample was derivatised with N-methyl-N-trimethylsilylheptafluor(o)butyramide. For GC-MS analysis, the quadrupole mass spectrometer MSD 5972A (Agilent, Santa Rosa, California, USA) was run in the selective ion-monitoring mode. The following characteristic mass fragment pairs were used for quantification: *m/z* 217/357 (5 α -cholestane, internal standard), *m/z* 329/368 (cholesterol), *m/z* 325/351 (7- and 8-dehydrocholesterol (DHC)), *m/z* 343/372 (desmosterol), *m/z* 255/458 (lathosterol), *m/z* 393/498 (lanosterol), and *m/z* 213/229 (8(9)-cholestenol).

Gas chromatography separation was achieved on a capillary column (DB-5MS, 30 m $\times$ 0.25 mm; film thickness: 0.25; J&W

Figure 3 Effect of cholesterol biosynthesis inhibitors on control lymphoblastoid cells (LCs) in the $[2\text{-}^{14}\text{C}]$ acetate loading test. Control LCs were preincubated on lipid-depleted cell culture medium for 24 h, and exposed to $1\text{ }\mu\text{M}$ AY9944, $1\text{ }\mu\text{M}$ triparanol, or $10\text{ }\mu\text{M}$ progesterone for 30 min before $2.5\text{ }\mu\text{Ci}$ $[2\text{-}^{14}\text{C}]$ acetate was added for 2 h. The LCs were then chased in lipid-depleted cell culture medium containing 144 mM sodium acetate for another 2 h. DHC, dehydrocholesterol.

Scientific, Folsom, California, USA) using helium as a carrier gas. A volume of $1\text{ }\mu\text{l}$ of the derivatised sample was injected in splitless mode.

Mutation analysis by direct sequencing

Genomic DNA was isolated from LC using standard methods. All coding exons and adjacent intron regions of the 7-dehydrocholesterol reductase (*DHCR7*) gene were sequenced with a capillary fluorescent sequencer (Applied Biosystems, Darmstadt, Germany). *DHCR7* genomic primer sequences are available on request.

Statistical analysis

To assess significant differences between sterol patterns in control and patient LC, non-parametric exact permutation tests were applied. Permutation tests are especially useful for small sample sizes (total number of observations <50) or single case designs. They have a relative asymptotic efficiency of one compared with parametric analogues (eg, *t* tests). Permutation tests incorporate all available numerical information (measurement values and sample sizes), but do not make any assumptions about the underlying distribution of the variables.^{20 21}

RESULTS

Separation of sterol standards

We developed a test system that separates C27–C30 sterols using TLC. Unmarked sterol standards (lanosterol, cholesterol, desmosterol, lathosterol and 7-dehydrocholesterol) were dissolved in ethanol, spotted on a 10% silver nitrate silica gel TLC plate with benzene/ethylacetate as mobile phase. Plates were dried and developed a second time in the same solvent, resulting in a higher retention fraction (RF) and a better separation. To visualise the sterol bands, the plate was sprayed with bromothymol blue. The smallest amount of sterol that could be visualised was $10\text{ }\mu\text{g}$, which exceeds the usual physiological concentrations of cholesterol precursor sterols. Because 8-DHC is not available commercially, we extracted the plasma sterols of a patient biochemically severely affected by

SLOS (7-DHC $665\text{ }\mu\text{mol/l}$, 8-DHC $373\text{ }\mu\text{mol/l}$, cholesterol $367\text{ }\mu\text{mol/l}$ by GC-MS analysis) and separated the dehydrosterols by TLC. The spots were scraped from the plate, extracted and analysed by gas chromatography-mass spectrometry (GC-MS). 7-DHC and cholesterol were detected in the positions known from the standards, while 8-DHC was found in the same position as desmosterol and lathosterol.

Influence of different organic solvents on sterol separation

Different ratios of benzene:ethylacetate were tested to identify the one allowing optimal separation of sterols, especially of cholesterol from desmosterol. Figure 2 shows that a sufficient separation of all sterols apart from cholesterol and desmosterol was obtained by benzene/ethylacetate 9/1 v/v and benzene/ethylacetate 5/1 v/v. Cholesterol and desmosterol could not be separated by any combination of the organic solvents. Higher concentrations of benzene caused lower RFs and poorer separation of lathosterol and desmosterol. Chloroform/acetone 27.6/1 for primary and diethyl ether/n-hexane 5/1 used for secondary development, which are described in the literature,²² resulted in the highest RF values, but adequate separation of cholesterol, desmosterol and lathosterol could not be obtained.

Inhibitors of cholesterol biosynthesis demonstrate sufficient separation of most $[2\text{-}^{14}\text{C}]$ sterols

The amount of sterol required to visualise non-radioactive sterols on the TLC plate greatly exceeds physiological concentrations. We therefore investigated whether a better separation could be achieved with the lower sterol concentrations used in our radioactive test system. To produce specific patterns of cholesterol precursors, we incubated control LC with three different inhibitors of cholesterol synthesis, AY9944, triparanol and progesterone, followed by $[2\text{-}^{14}\text{C}]$ acetate loading (fig 3). The resulting sterol patterns without radioactive labelling were analysed in parallel by GC-MS.

Triparanol is an inhibitor mainly of sterol- $\Delta 24$ reductase, causing an accumulation of desmosterol, which was demonstrated in both test systems. At higher concentrations of triparanol, sterol- $\Delta 8$ -isomerase is also blocked,²³ causing an increase in 8(9)-cholestenol and 8-DHC levels. This inhibition was only seen in the more sensitive $[2\text{-}^{14}\text{C}]$ acetate loading test, with the corresponding bands located directly above 7-DHC and directly below desmosterol/lathosterol. A sufficient separation of cholesterol and desmosterol and of desmosterol and 8-DHC in the radioactive test, when the amounts were not much greater than physiological concentrations, was therefore confirmed.

AY9944 inhibits sterol- $\Delta 7$ reductase. The resulting accumulation of 7-DHC and, to a far lesser degree, 8-DHC¹¹ as well as reduced cholesterol synthesis were clearly shown in both test systems, but were more evident in the loading test. Higher concentrations of AY9944 inhibit sterol- $\Delta 8$ -isomerase,²⁴ which could be shown in both systems.

Progesterone inhibition, expected to block sterol- $\Delta 24$ reductase at a concentration of $10\text{ }\mu\text{M}$,²⁵ caused diminished cholesterol synthesis and moderate increases in desmosterol, which were not as prominent as with triparanol. Slight increases in all identified sterols and the occurrence of additional unknown bands are in accordance with a possible role of progesterone in the inhibition of intracellular sterol transport.²⁶

Because the $[2\text{-}^{14}\text{C}]$ acetate loading test labels only newly synthesised sterols, the sterol quantification differs from the GC-MS analysis, which represents all sterols in the cells (table 1). The location of the radioactive sterol bands corresponds to those of the non-radioactive standards.

Table 1 Quantification of sterols after exposure of control cells to inhibitors of cholesterol biosynthesis: comparison between gas chromatography-mass spectrometry and the [2-¹⁴C]acetate loading test

	Controls (n = 9)			Controls+inhibitors (n = 2)		
	Median	Range	Mean (SD)	Tripalanol 1 μM	AY9944 1 μM	Progesterone 10 μM
GC-MS						
Cholesterol	94.3	92.5–97.5	94.7 (1.6)	94.3	74.7	92.9
Desmosterol	0.79	0.32–1.72	0.84 (0.4)	3.25	0.45	4.45
Lathosterol	1.42	0.34–1.99	1.36 (0.5)	1.40	3.76	1.76
7-DHC	0.41	0.16–0.55	0.41 (0.1)	0.60	18.95	0.57
8-DHC	0.2	0.13–0.46	0.24 (0.1)	0.20	1.31	0.21
8(9)-Cholestenol	<dl	<dl	<dl	<dl	1.34	<dl
Lanosterol	0.13	0.01–1.72	0.32 (0.5)	0.18	0.08	0.10
[2- ¹⁴ C]acetate loading						
Cholesterol	82	74–90	82 (5)	28	5	19
Desmosterol/lathosterol	8.2	4.4–10.6	7.8 (1.9)	22	16	16
7-DHC	1.9	1.0–3.4	2.0 (0.8)	12	39	34
8-DHC	<dl	<dl	<dl	9	14	9
8(9)-Cholestenol	<dl	<dl	<dl	21	21	11
Lanosterol	4.7	2.3–7.9	4.7 (1.9)	7	6	10

DHC, dehydrocholesterol; dl, detection limit; GC-MS, gas chromatography-mass spectrometry.

GC-MS analysis was performed after cultivation of the cells on lipid-depleted culture medium for 48 h and exposure to each inhibitor for 72 h. The [2-¹⁴C]acetate loading test was performed as described in the legend of fig 3. Data are expressed as percentage of total sterols.

However, [2-¹⁴C]desmosterol and [2-¹⁴C]lathosterol could not be separated by the system. Because an increase of each component would result in an abnormal pattern and differentiation could be achieved later by GC-MS, we considered this sufficient for a screening test.

Cholesterol metabolism in human LC lines

We next investigated the sterol pattern in LC lines from 228 patients with HPE using the [2-¹⁴C]acetate loading test. The amount of newly synthesised radioactive sterols was compared with controls (n = 9, table 1). An abnormal sterol pattern was

Table 2 Incorporation of [2-¹⁴C]acetate into cellular sterols and gas chromatography-mass spectrometry sterol analysis of lymphoblastoid cell lines from a patient with known Smith-Lemli-Opitz syndrome and from 22 patients with holoprosencephaly showing an abnormal sterol pattern in the [2-¹⁴C]acetate loading test

	Cholesterol		Desmosterol		Lathosterol		7-DHC		8-DHC		Lanosterol		DHCR7 mutation
	¹⁴ C acetate loading	GC-MS	¹⁴ C acetate loading	GC-MS	¹⁴ C acetate loading	GC-MS	¹⁴ C acetate loading	GC-MS	¹⁴ C acetate loading	GC-MS	¹⁴ C acetate loading	GC-MS	
Controls	72–91	91–98	11.7	1.7	11.7	2.4	3.5	0.64	11.7	0.4	8.5	1.3	
Patient													
SLOS	4.9	60	25	0.1	25	4.7	61	32	25	3.7	8.0	0.1	R352W/IVS8-1G>C
144	63	91	15	2.3	15	2.1	4.8	0.6	15	0.3	8.9	1.9	ND
329	60	95	16	0.9	16	1.9	9.6	0.4	16	0.2	7.4	0.3	–/–
331	36	94	37	0.7	37	3.6	13	0.9	37	0.4	8.4	0.1	–/–
383	68	93	13	0.4	13	5.1	6.9	0.8	13	0.4	3.3	<dl	–/–
613	19	72	23	0.1	23	3	47	22	23	1.8	6.3	0.2	T154R/G410S
646	34	97	38	<dl	38	1.6	11	0.8	38	0.2	8.6	<dl	ND
719	62	95	17	0.3	17	2.0	5.4	0.5	17	0.3	8.0	<dl	–/–
811	76	94	11	0.4	11	1.8	5.8	1.4	11	0.2	3	<dl	–/–
859	37	92	21	5.4	21	1	11	0.7	21	0.7	22	<dl	ND
887	56	94	19	0.8	19	2.1	6.2	0.4	19	0.2	7	0.4	–/–
893	64	91	18	0.3	18	5.8	2.8	0.9	18	0.5	9.2	0.1	ND
1265	63	93	14	0.2	14	0.9	14	0.9	14	1.5	6.9	<dl	–/–
6006	48	94	18	0.4	18	1.3	19	1.6	18	0.4	3.9	<dl	–/–
6833	75	95	11	0.4	11	2.2	7.9	0.4	11	0.2	3.2	0.4	–/–
7304	58	94	21	0.9	21	1.9	11	0.5	21	0.2	5.4	0.5	–/–
7334	77	94	7.6	0.4	7.6	2.1	5.6	0.5	7.6	0.4	5.9	0.1	R242H/–
7835	66	96	14	0.6	14	1.6	12	0.9	14	0.3	4.9	0.4	–/–
7858	74	93	10	0.4	10	0.8	11	0.3	10	0.2	2.6	0.2	–/–
7880	51	91	18	0.5	18	1.7	12	0.3	18	0.2	5.1	0.3	–/–
8064	61	94	18	0.9	18	1.5	5.9	0.7	18	0.4	8.6	0.5	ND
8103	42	97	22	1.1	22	0.7	14	0.3	22	0.2	8.6	0.3	ND
8121	66	93	14	0.3	14	1.3	13	0.3	14	0.1	2.7	0.3	–/–

DHC, dehydrocholesterol; DHCR, 7-dehydrosterol reductase; GC-MS, gas chromatography-mass spectrometry; ND, not detected; SLOS, Smith-Lemli-Opitz syndrome. Indicated is the percentage of total sterols, mean of three independent experiments ([2-¹⁴C]acetate-loading) or two independent experiments (GC-MS). The control values represent mean (2 SD) of nine controls (cholesterol) or mean (2 SD) (cholesterol precursors). Data are printed in bold if they fell below this range (cholesterol) or exceeded this range (cholesterol precursors).

Table 3 Clinical characterisation of patients with an abnormal sterol pattern in lymphoblastoid cells

Patient	Age at diagnosis	Gender	Mutation	Cerebral malformation	Facial malformation	Additional clinical features	Severity	Reference
144	1 day	F		Alobar HPE, ACC, arhinencephaly, Chiari I malformation	Dysplastic ears, wide nasal bridge, high narrow palate	Lumbosacral MMC	Severe	
329	3 months	F	PTC: T728M	Partial ACC	Midline CLP, SCL, hypotelorism, flat midface	Omphalocele, ambiguous genitalia, panhypopituitarism, PDA	Severe	Ming 2002 ²⁷
331	3 days	F		Semilobar HPE, hypoplastic CC, pituitary gland absent	Microcephaly, midline CLP, hypotelorism, up-slanted palpebral fissures	ASD, PDA, vascular malformation, talipes equinovarus, NEC, anal malformation, epilepsy	Moderate-severe	
383	1 month	F		Semilobar HPE	Microcephaly, Robin sequence, preauricular tag	VSD, ASD, partial syndactyly toes 2,3,4 r	Moderate	
613	2 months	F	DHCR7: T154R/G410S	Semilobar HPE, cebocephaly	Microcephaly, median CLP, hypotelorism, ptosis, single nostril	Partial bilateral syndactyly toes 2+3, polydactyly	Moderate-severe	Kelley 1996 ¹²
646	Adult	F	46, XX, t(10;13)(p15;q23)	Normal brain	Normal face	Two sons with alobar HPE	Asymptomatic	
719	2½ years	F		Lobar HPE, partial ACC, singular ventricle	Microcephaly, hypotelorism, SCL, microphthalmia	DI, infantile spasms, spastic tetraparesis	Mild-moderate	
811	Adult	M		Normal brain	Normal face	Son with semilobar HPE	Asymptomatic	
859	1 day	M	46, XY, r (7); del SHH	Semilobar HPE	Microcephaly, median CLP, hypotelorism, ptosis, single nostril	Micropenis, rugate scrotum, hypoplastic nipples, intractable seizures	Severe	Sawyer 1996 ²⁸
887	7 years	F		Semilobar HPE, partial ACC, hydrocephalus, IH cyst		Mental retardation	Mild-moderate	
893	2½ months	F	dup ZIC2	Semilobar HPE	Normal face	None	Not known	
1265	20 months	F	SIX3: L226V	Normal brain	Microcephaly, hypotelorism, midface hypoplasia, absence of nasal bones, bilateral CLP	None	Mild	Wallis 1999 ⁵
6006	1 month	F		Alobar HPE, hydrocephalus	Hypotelorism, midface hypoplasia, small nose, submucous CP	Seizures	Moderate	
6833	3 months	M	ZIC2: 552-568 dup16	Semilobar HPE	Microbrachycephaly, narrow palate	DI, muscular hypertonia, severe psychomotor retardation, recurrent aspiration	Moderate-severe	
7304		F						
7334	4 months	F		HPE			Not known	
7835	9 months	M		HPE	SCL, nasal pyriform aperture stenosis		Mild	
7858	5 months	F		HPE			Not known	
7880	20 months	F		Semilobar HPE			Mild	
8064	5 months	F		Semilobar HPE			Mild	
8103		F						
8121	4 months	F		Normal brain	Microcephaly, hypotelorism, SCL, low-set ears, bilateral cataracts	IUGR, oligohydramnios, 2/3 toe syndactyly, hypopituitarism, mental retardation	Mild	

ACC, agenesis of corpus callosum; ASD, atrial septal defect; CC, corpus callosum; CLP, cleft lip palate; CP, cleft palate; DI, diabetes insipidus; HPE, holoprosencephaly; IH, intrahemispheric; IUGR, intrauterine growth retardation; MMC, myelomeningocele; NEC, necrotising enterocolitis; PDA, patent ductus arteriosus; SCL, single central incisor; VSD, ventricular septal defect.

defined if the percentage of total sterols exceeded (cholesterol precursors) or fell below (cholesterol) 2 SD of the control mean. Because 8(9)-cholesterol is undetectable in controls, any amount was considered abnormal. GC-MS sterol analysis was performed in all cell lines with an abnormal sterol pattern in the [2-¹⁴C]acetate loading test. Experiments were performed either in triplicate ([2-¹⁴C]acetate loading test) or in duplicate (GC-MS).

Abnormal sterol patterns were found in 22 patients (9.6%) by [2-¹⁴C]acetate loading (table 2). Mean (SD) cholesterol levels

as a fraction of total sterols in these patients were 57% (15.3%), whereas they were 82% (4.7%) in controls ($p < 0.001$). Mean (SD) 7-DHC levels were 11.3% (8.9%) in patients and 2% (0.8%) in controls ($p < 0.001$). Mean (SD) desmosterol/lathosterol/8-DHC levels were 18% (7.5%) in patients and 7.8% (1.9%) in controls ($p < 0.001$). Mean (SD) lanosterol levels were not significantly different in patients and controls: patients 6.8% (4.0%) and controls 4.7% (1.9%, $p = 0.16$). Notably, nine of the patients with abnormal loading test results had normal sterol profiles by GC-MS.

Patient 613 displayed the typical SLOS pattern both by [2-¹⁴C]acetate loading and by GC-MS, notably a marked increase in 7-DHC. GC-MS analysis additionally showed a moderate increase in 8-DHC and decrease in cholesterol. There was a mild, possibly non-specific, increase in lathosterol. In the [2-¹⁴C]acetate loading test, there was nearly no newly synthesised cholesterol, reflecting the severity of biochemical impairment. Surprisingly, in this patient and in a patient with known SLOS (SLOS-positive control in table 2), 8-DHC could not be separated from the desmosterol/lathosterol band. The moderately increased desmosterol/lathosterol/8-DHC band therefore likely reflects an accumulation of 8-DHC. Patient 613 had two different mutations in *DHCR7*, confirming the diagnosis of SLOS. Because 7-DHC was the prominent sterol in 14 other cell lines, we excluded SLOS in them by mutation analysis (table 2). In the remaining patients, [2-¹⁴C]acetate loading revealed an abnormal sterol pattern with equal increases in several sterols and a reduction in cholesterol in most patients. Table 3 lists the clinical features of all patients with an abnormal sterol pattern.

DISCUSSION

In an important investigation into the biochemical basis of HPE in SLOS, Cooper *et al*⁹ showed that abnormal SHH signalling in SLOS was caused by decreased cellular levels of cholesterol, not by the increase of any cholesterol precursor. Because SLOS represents only one of many genetic disorders that should manifest decreased tissue levels of cholesterol, we developed a sensitive sterol-labelling assay to detect both mild enzymatic defects in post-squalene cholesterol biosynthesis and disorders in which the dominant abnormality will be only a decreased rate of cholesterol synthesis. We previously described this assay as a sensitive and rapid method for diagnosis of SLOS in human fibroblasts even in biochemically mildly affected patients.²⁹ In lymphoblasts of a patient with known moderately affected SLOS, the assay shows an accumulation of 7-DHC and desmosterol/lathosterol/8-DHC and a massively reduced cholesterol synthesis (table 2; patient with SLOS). We then applied this new assay to 228 HPE cell lines, in which we expected to find a variety of abnormalities of cholesterol homeostasis.

In 22 of 228 patients with HPE, we found impaired cholesterol biosynthesis. In all, 18 of these 22 patients had reduced incorporation of [2-¹⁴C]acetate into cholesterol, and all had abnormal levels of one or more different cholesterol precursors (table 2). Notably, nine of these patients had normal GC-MS results, which we interpret as a greater sensitivity of the [2-¹⁴C]acetate loading test, which specifically displays newly synthesised sterols under unfavourable metabolic conditions, whereas the GC-MS method measures total cellular sterols. Nevertheless, GC-MS is indispensable to clearly identify the accumulated sterols, because desmosterol, lathosterol and 8-DHC cannot be separated by the TLC method.

In addition to one patient with a SLOS pattern and confirmed mutations in *DHCR7* (T154R/G410S), we found an isolated accumulation of 7-DHC in four patients and a combination of increased 7-DHC and desmosterol/lathosterol/8-DHC in 11 others. Because we could not determine which of the three latter sterols was increased, SLOS was at first a possible diagnosis in all patients. However, SLOS was excluded in all by molecular analysis (table 2).

Five patients had alterations in known HPE genes in addition to their sterol abnormalities. Patient 859 had a midline cleft palate and a ring chromosome 7 with 7q36.3 deletion including *SHH* and an accumulation of lanosterol, a C30 sterol, in the [2-¹⁴C]acetate loading test. This observation is consistent with possible haploinsufficiency for *INSIG1*, which is located at 7q36.3, close to *SHH*. *Insig*-deficient mouse embryos have

midline facial clefting and accumulate lanosterol and other cholesterol precursors,³⁰ presumably on the basis of enhanced degradation rates for enzymes distal to lanosterol synthase. In patient 329, a *PTC* T728M mutation was found, which is located between transmembrane domains 6 and 7. This domain is predicted to be a cytoplasmic loop with yet unknown functional relevance. It is not associated with the sterol-sensing domain (SSD_5TM; IPR000731) in the patched protein, so a primary effect of this mutation on sterol levels is unlikely. In patient 646, a t(10;13)(p15;q23) translocation was identified. Even though we have not studied the translocation breakpoints in detail, it is of theoretical interest that *ID11* is located in 10p15. *ID11* encodes a peroxisomally localised enzyme that catalyses the interconversion of isopentenyl diphosphate to dimethylallyl diphosphate, an early step in cholesterol biosynthesis. Although a defect in the pre-squalene pathway could impair overall cholesterol synthesis and cause the reduced incorporation of [2-¹⁴C]acetate into cholesterol, the observed accumulation of cholesterol precursors could not be explained. There is no evidence for sterol regulatory genes in 13q22.3–13q32.

In the remaining five patients (144, 646, 893, 8064 and 8103), nearly all cholesterol precursors were increased to the same amount, whereas cholesterol synthesis was variably reduced, so that a single enzyme block was unlikely. In earlier studies, it was reported that the cholesterol precursors desmosterol, lathosterol, lanosterol, zymosterol and 7-DHC are transported from their site of synthesis in the endoplasmic reticulum (ER) to the cell membrane.^{31–34} There is evidence that the transport process of these sterols differs from that of newly synthesised cholesterol and from each other.³⁴ After arrival at the cell membrane, sterol precursors are either effluxed to high-density lipoprotein and phosphatidylcholine vesicles,³¹ or transported back to the ER for conversion to cholesterol.³⁵ Disruption of sterol transport from the ER to the plasma membrane or back to the ER will cause an accumulation of one or more cholesterol precursors within the cell and in a reduced availability of cholesterol. We therefore speculate that an impaired intracellular transport of cholesterol precursors and the resulting decrease in cholesterol could alter SHH signalling in these patients.

Taken together, our data suggest that an accumulation of sterol intermediates caused either by defective regulation of cholesterol biosynthesis or by defects of intracellular sterol transport could further aggravate impaired SHH signalling, leading to a severe malformation syndrome. A detailed analysis of cholesterol biosynthesis and homeostasis should therefore be considered in all patients with HPE.

ELECTRONIC DATABASE INFORMATION

Bioinformatic Harvester (<http://harvester.embl.de>); InterPro (<http://www.ebi.ac.uk/interpro>); Entrez Gene (<http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?db=gene>); UCSC Proteome Browser (<http://genome.ucsc.edu/cgi-bin/pbGateway>).

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