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ARTICLE

New insights into genotype–phenotype correlation for *GLI3* mutations

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The phenotypic spectrum of *GLI3* mutations includes autosomal dominant Greig cephalopolysyndactyly syndrome (GCPS) and Pallister–Hall syndrome (PHS). PHS was first described as a lethal condition associating hypothalamic hamartoma, postaxial or central polydactyly, anal atresia and bifid epiglottis. Typical GCPS combines polysyndactyly of hands and feet and craniofacial features. Genotype–phenotype correlations have been found both for the location and the nature of *GLI3* mutations, highlighting the bifunctional nature of GLI3 during development. Here we report on the molecular and clinical study of 76 cases from 55 families with either a *GLI3* mutation (49 GCPS and 21 PHS), or a large deletion encompassing the *GLI3* gene (6 GCPS cases). Most of mutations are novel and consistent with the previously reported genotype–phenotype correlation. Our results also show a correlation between the location of the mutation and abnormal corpus callosum observed in some patients with GCPS. Fetal PHS observations emphasize on the possible lethality of *GLI3* mutations and extend the phenotypic spectrum of malformations such as agnathia and reductional limbs defects. *GLI3* expression studied by *in situ* hybridization during human development confirms its early expression in target tissues.
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INTRODUCTION

Mutations in the *GLI3* gene lead to several clinical phenotypes including Greig cephalopolysyndactyly syndrome (GCPS; MIM# 175700)[1] and Pallister–Hall syndrome (PHS; MIM# 146510).[2] PHS first described in 1980 by Hall as a lethal condition in neonatal period[3,4] associates mainly hypothalamic hamartoma (HH), postaxial polydactyly (PD), bifid epiglottis and imperforate anus (IA). PHS forms a spectrum from very mild cases with subtle insertional PD to severe cases.[5] Typical GCPS is characterized by polysyndactyly in hands and/or feet, craniofacial abnormalities, such as macrocephaly and hypertelorism,[6] and developmental delay in individuals with large deletions encompassing *GLI3*.[7]

GCPS and PHS are distinct entities both caused by mutations in the transcription factor *GLI3*, a modulator of *Sonic hedgehog* (SHH) pathway with a bifunctional nature, either activator or repressor. In the presence of SHH, full-length GLI3 functions as a transcriptional activator (GLI3A), whereas in the absence of SHH, GLI3 is cleaved to produce a repressor (GLI3R).[8]

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Previous reports demonstrate a robust genotype–phenotype correlation of *GLI3* mutations.[9,10] Truncating mutations in the middle third of the gene generally cause PHS, resulting in a constitutive repressor protein. By contrast, haploinsufficiency resulting from chromosomal rearrangements, but also missense, splicing or truncating mutations elsewhere in the gene cause GCPS by loss of the DNA-binding capacity[11] or activation of nonsense-mediated mRNA decay,[12] or by the formation of an unstable or mislocalized protein.[13,14]

In this study, we report on the clinical and molecular data of a French cohort of 76 individuals from 55 families carrying a *GLI3* molecular defect. Most of mutations are novel and consistent with the previously reported genotype–phenotype correlation. In addition, our results also show a correlation between the location of the mutation and corpus callosum dygenesis observed in some GCPS individuals. Fetal observations emphasize on the possible lethality of *GLI3* mutations, extend the phenotypic spectrum of malformations to severe craniofacial and reductional limb defects. *GLI3* expression studied by *in situ* hybridization during human development confirms its early expression in target tissues including in pharyngeal arches, and later in mandible.

PATIENTS AND METHODS

Patients

Index cases were tested for mutations in the *GLI3* gene because of the presence of clinical findings compatible with the diagnosis of GCPS or PHS and 76 cases from 55 families are included in this upon identification of a *GLI3* molecular defect.

A total of 55 patients from 38 families with features compatible with GCPS (polysyndactyly in hands and/or in feet and/or dysmorphic features associating high forehead, macrocephaly or widely spaced eyes and/or corpus callosum anomalies) were identified with a *GLI3* mutation or rearrangement, 39 cases were familial and 16 sporadic.

Also, 21 PHS individuals from 17 families with postaxial or insertional PD and/or HH with pathogenic *GLI3* mutations have been included. Among them, 13 were sporadic, 7 familial and 1 of unknown inheritance. Antenatal cases were selected for either HH or IA plus at least 2/5 features belonging to the PHS spectrum namely intrauterine growth retardation (IUGR), limb malformation, heart disease, micropenis and renal anomaly. In all seven fetuses with *GLI3* mutation, pregnancy was terminated because of severe clinical findings, in accordance with French legislation. A written informed consent for genetic analysis was obtained from each family before testing, and for autopsy in all fetal cases.

Molecular genetic studies

GLI3 mutation screening. Genomic DNA was extracted from frozen fetal tissue or amniocytes for the fetal cases and from peripheral blood samples in the postnatal cases. The 14 coding exons and the adjacent intronic regions of the *GLI3* gene were amplified using *GLI3*-specific primers pairs (available on request). Direct sequencing of PCR products was performed using the Big Dye Terminator Cycle Sequencing Kit v3 (Applied Biosystems, Courtaboeuf, France) and analyzed on an ABI3130 automated sequencer (Applied Biosystems). Sequences were analyzed with Seqscape software v2.5 (Applied Biosystems). Sequence data were compared with the *GLI3* reference sequence NM_000168.5, and mutation named according to the HGVS nomenclature and checked by the *Mutalyzer* programme.[15] Mutations have been submitted to the public database LOVD.

All missense variants identified were investigated by *in silico* analysis using SIFT and PolyPhen2. Parental studies were done in sporadic cases to confirm a *de novo* occurrence of the alterations when parental DNA was available. We classified novel variants as pathogenic mutations the nonsense, frameshift and splice variants, and the missense variant, which affected conserved nucleotide or amino acid, segregating with the disease in familial cases, and/or apparently *de novo* in sporadic cases. Confirmation of biological parentage was performed for the *de novo* missense mutation only.

GLI3 rearrangement analysis. Individuals without *GLI3* coding sequence mutations underwent fluorescent *in situ* hybridization (FISH), Multiplex Ligation-dependent Probe Amplification (SALSA MLPA KIT P179 Limb Malformations-1—MRC-Holland, Amsterdam, The Netherlands) or Array comparative genomic hybridization (Array CGH Agilent 244 or 180K oligonucleotide microarrays, Agilent Technologies, Santa Clara, CA, USA).

Gene expression analyses using *in situ* hybridization

Human embryos and fetal tissues were obtained from legally terminated pregnancies in agreement with French law (94–654 of 29 July 1994), following National Ethics Committee recommendations and with approval from the Necker Hospital ethics committee. Five developmental stages using Carnegie staging (CS)[16] were studied: C14, C16, C18, C19 and 8.5 weeks of development. Tissues were fixed in 4% phosphatase-buffered paraformaldheyde, dehydrated and embedded in paraffin blocks, and 5 μm-thick serial sections were cut. Exon 3 primers were selected for PCR amplification (3F-TACTTCTTTTCCGGGAGAGG and 3R-CCATAGCTC CTGAACAAGTG). Sense and antisense riboprobes were generated using either T7 or T3 RNA polymerase. Riboprobe labeling, tissue fixation, hybridization and developing were carried out according to standard protocols as described previously.[17] No hybridization signal was detected with the labeled sense probe, confirming that the expression pattern obtained with the antisense probe was specific. Adjacent slides were hematoxylin/eosin stained for morphological studies.

RESULTS

Detailed clinical phenotypes and molecular results are described in Table 1 (GCPS) and Table 2 (PHS). Frequencies reported in Tables 3 and 4 are represented as the ratio between the number of patients with a particular finding and the total number of patients for which the information was available.

GLI3 mutations and deletions

Greig cephalopolysyndactyly syndrome. We identified 32 causative mutations and 6 large deletions in 38 GCPS index cases. Among the 16 sporadic cases, the *de novo* occurrence in the proband was confirmed for 6 patients after analysis of both parents. Three mutations were recurrent (c.1874G>A, c.4463del and c.444C>A identified each in two families). Overall, 8/38 (21%) were nonsense mutations, 17/38 (45%) were frameshift mutations predicting a premature stop codon, 6/38 (16%) were missense mutations, 1/38 (3%) was a splice mutation and 6/38 (16%) were complete deletions of the gene. Nine of them were located in the N-terminal part of GLI3 before the zinc-finger domain predicting a prematurely terminated protein lacking the DNA-binding domain. Interestingly, all 6 missense mutations were within DNA-binding domain extending from amino-acid 462 to 645 encoded by exons 10 to 13 of the *GLI3* gene (Figure 1). All six missense mutations are predicted to be probably damaging to the protein function *in silico* by both PolyPhen-2 and SIFT softwares, involving conserved amino acids. Ten truncating mutations are located in the 1/3 end of the protein, within the transactivation domains TA2 and TA1.[11] Interestingly, two truncating mutations (c.2082_2083delinsAGAGAAGCC and c.3427_3443del) were in the previously defined PHS region (between cDNA positions 1998 and 3481).[9] Among the 29 different mutations found in this series, two (c.868C>T and c.1874G>A) were previously described in other patients[9] and 27 are novel mutations. Of note, a frameshift mutation (c.1543_1544dup) found in two affected sibs, was present at low level in DNA extracted from blood of their father (Family G068), suggesting a somatic mosaicism. Along the same line, a FISH analysis revealed a *GLI3* deletion in only 56% of blood cells of a patient (G059) with bilateral preaxial PD of the feet and developmental delay. At least two patients (G005 and G019) had Greig cephalopolysyndactyly contiguous gene syndrome (GCPS-CGS)

Table 1 Clinical features and *GLI3* molecular results in GCPS cases

N° (#)	cDNA alteration (c.)	Predicted protein alteration (p.)	Inheritance	Postaxial PD	Preaxial PD	Broad thumbs or halluces	Syndactyly	Macrocephaly	Widely spaced eyes	MRI Findings	Developmental delay	Additional findings
G029	327del	Phe109Leufs*50	F	–	FB		+	–	–	–	–	Precocious puberty, scaphocephaly
G070	427G>T	Glu143*	F	HB	FL		–	+			–	
Mother	427G>T	Glu143*	F	–	–		–	–	–		–	
G118	444C>A	Tyr148*	F	–	FB	BT	+	+			–	
G13684	444C>A	Tyr148*	F	–	FB		+	+	+		–	
Brother	444C>A	Tyr148*	F	–	FL		+	–	+		–	
Mother	444C>A	Tyr148*	F	–	FB		+	+			–	
G099	518dup	Ile174Hisfs*2	F	–	FB		+	+	–		–	
G048	679+1G>T	Splice	*De novo*	HB	FB, HB					VD		Delta phalanx
G15198	833_843del	Arg278Thrfs*22	F	HB, FB	FB, HB	BT	+	+	+	–	–	
G079	868C>T	Arg290*	*De novo*	HB	FB		+	+	+		–	Atrial septal defect
G088	997_998dup	Tyr334Profs*14	*De novo*	–	FB	BT	+	+	+		–	Umbilical hernia
G14083	1063_1067dup	Leu357Serfs*10	*De novo*	–	FB, HB	BT	+	–	–			Bifid distal phalanx, BW= 4150
G033	1378del	Val461Serfs*41	F	–	FB	BT	+	+	+		–	
G076	1498C>T	His500Tyr	F	–	FB		+	+	–	+	+	Cerebral prematurity sequelae
Mother	1498C>T	His500Tyr	F	–	FB		+	–	–		–	
Brother	1498C>T	His500Tyr	F	HB	FB		+	–	–		–	
G068	1543_1544dup	Arg516Alafs*20	F	–	FB		+	–	–		–	Delta metacarpal, BW=4880
Brother	1543_1544dup	Arg516Alafs*20	F	–	FB		+	–	–		–	BW=4740
Father	[=/1543_1544dup]	[=/Arg516Alafs*20]	F	–	–		–	–	–		–	
A018	1733G>C	Cys578Ser	S	HB	FB	BT	+	+	+	CCH, VD		Hypoplastic cerebellum, microretrognathism
G094	1745del	Gly582Valfs*47	F	HB, FB	–			+	–		–	
G16012	1767del	Asn589Lysfs*40	F	–	FB		+	–	–		–	Bilateral keratoconus, umbilical and bilateral inguinal hernia
Daughter	1767del	Asn589Lysfs*40	F	–	–	BT	+	–	–		–	Umbilical hernia
G109	1786C>T	His596Tyr	F	HB	FB		–	–	+			Macrosomia
Father	1786C>T	His596Tyr	F	–	–	BH	–	–	–		–	
Sister	1786C>T	His596Tyr	F	–	FB		+	–	+			
G056	1787A>C	His596Pro	F	–	FB		+	+	+		+	Neurofibromatosis type 1
Father	1787A>C	His596Pro	F	–	FB		+	+			–	
G026	1874G>A	Arg625Gln	S	–	FB, HB		+	–	–		–	Brachydactyly, delta phalanx
G14331	1874G>A	Arg625Gln	*De novo*	–	FB	BT, BH	+	+	+			Brachydactyly, speech delay
G111	2082_2083delinsAGAGAAGCC	Val695Glufs*45	F	HB	FB		–	–	–		–	
G063	3427_3443del	Phe1143Alafs*98	F	–	FB		–	–	+	VD	+	Speech delay, exomphalos
G003	3559C>T	Gln1187*	F	HB	–		–	+	+		MD	
Brother	3559C>T	Gln1187*	F	HB, FB	–	BT	–	+	+		–	
Mother	3559C>T	Gln1187*	F	HB	–		–	–			–	

Table 1 (Continued)

N° (#)	cDNA alteration (c.)	Predicted protein alteration (p.)	Inheritance	Postaxial PD	Preaxial PD	Broad thumbs or halluces	Synd-actyly	Macro-cephaly	Widely spaced eyes	MRI Findings	Developmental delay	Additional findings
G051	3950del	Pro1317Glnfs*102	F	HB	–		–	+	–	pCCA	Mild	
Mother	3950del	Pro1317Glnfs*102	F	HB	FB		–	+	–			
A019 F*	4099dup	Ala1367Glyfs*45	S	HB, FB	–		+	–	+	pCCA		
G004	4324C>T	Gln1442*	S	HB, FB	–	BT	–	+	+	pCCA	+	Umbilical hernia, anterior anus
G002	4408C>T	Gln1470*	F	HB, FB	–		+	+	+	CCH, VD	–	Laryngomalacia
G006	4431dup	Glu1478*	F	HB, FB	–		–	+	+	CCH		BW = 4440
A017	4456C>T	Gln1486*	S	HB, FB	–		–	+	–	CCH	–	
G016	4463del	Thr1488Lysfs*23	*De novo*	–	FB		+	+	–	CCA, VD	Fine	
G043	4463del	Thr1488Lysfs*23	F	HB, FB	FB		+	+	–		Mild	Supernumerary nipples
Father	4463del	Thr1488Lysfs*23	F	HB, FB	FB		–	+	–			
Brother	4463del	Thr1488Lysfs*23	F	HB, FB	FR		–				Mild	
G114	4615_4624del	Thr1539Glyfs*11	F	HB	–	BH	–	+	–		–	
Sister	4615_4624del	Thr1539Glyfs*11	F	HB	FB		+	+	–		–	
G019	chr7.hg19:g.(35 674 000_37 280 000)_(46 116 000_46 598 000)del	*De novo*	–	FB		+	+	+	Thin CC	+	Bilateral inguinal hernia, strabismus	
G005	chr7.hg19:g(38521704_45810267)del	*De novo*	–	FB	BT	+	–	+	+	DD	Cataract, strabismus, vermis dysgenesis	
G028	rsa7p14.1(kit P179)x1	S	–	FB	BT	+	+		VD	DD	Seizures, strabismus, horseshoe kidney, BW = 4280	
G115	46,XY.ish del(7)(p14.1)(RP11-816F16-)	F	HB	FB		+	+	–	–	–		
G038	46,XX.ish del(7)(p14.1p14.1)(GLI3-)	S	–	FB		+	+	–	VD	MD		
G059	46,XY.ish del(7)(p14.1)(GLI3-)[56]/7p14.1(GLI3x2)[44]	S	–	FB		–	–	–	–	DD	Trigonocephaly, macrosomia	

Abbreviations: BH, broad halluces; BT, broad thumb; BW, birth weight; CCA, corpus callosum agenesis; CCH, corpus callosum hypoplasia; DD, developmental delay; F, familial; FB, feet bilateral; FR, foot right; FL, foot left; F*, fetal case; HB, hand bilateral; MD, motor delay; pCCA, partial CCA; PD, polydactyly; S, sporadic; VD, ventricular dilatation. #: Index cases are indicated in bold and related are below.

Table 2 Clinical features and *GLI3* molecular results in PHS cases

N°	cDNA alteration (c.)	Predicted protein alteration (p.)	Inheritance	Growth delay/GH deficiency	Insertional/ postaxial PD	Brachytelephalangism/ dactyly	Y-shaped metacarpal/ metatarsal	Hypothalamic hamartoma	Craniofacial anomalies	Anal atresia	Bifid epiglottis	Cardiac anomalies	Renal anomalies	Genital anomalies	Lung dysplasia	Intellectual deficiency	Nail dysplasia	Other findings
P15112	1995del	Gly666Alafs*27	*De novo*	–	–	+	+	+	+	+	+	–	–	–	–	–	+	Overlapping toes, preauricular tag
G097	2072del	Gln691Argfs*2	*De novo*	+	+		+	+	–	–		–	–	+	–	–		Micropenis, thin CC
G085	2123_2126del	Gly708Valfs*24	Familial	–	+		+		–	–		–		–		–	+	
Father	Gly708Valfs*24	Gly708Valfs*24		+	+		+		–	–		–		+		–		Micropenis
Aunt	Gly708Valfs*24	Gly708Valfs*24			+	+			–	–		–		–		–	+	
Grand-mother	Gly708Valfs*24	Gly708Valfs*24			+				–	–		–		–		–	+	
G121	2149_2150insT	Gln717Leufs*21	?	–	+		+		–	–		–	–	–		–	–	
G001	2149C>T	Gln717*	*De novo*	+	+	+	+	+	–	+		–	+	+		–		Sacrococcygeal teratoma, conical teeth, cryptorchidism, micropenis, syndactyly, unilateral renal agenesis, fine motor delay
G083	2385del	Ile796fs*13	Familial	–	+		+		–	–		–	–	–		–	–	
Father	2385del	Ile796fs*13		–	+		+		–	–		–	–	–		–	–	
G082	2641_2642dup	Gln881Hisfs*10	*De novo*	+	+	+	+	+		+		+	–	+		+	–	Micropenis, choanal atresia, IVC, fine DD
G044	2647G>T	Glu883*	Familial	+	+	+	+	+	–	–	–	–	–	–		–	+	Scoliosis, dental malposition
G072 F*	2685C>G	Tyr895*	*De novo*	–	+	+		+	–	–	+	–	–	–	–			Oligohydramnios, syndactyly
GBUL	2799del	Tyr933*	*De novo*	+	+	+	+	+	–	+		–	+	+		+	+	Seizures, panhypopituitarism, DSD (karyotype 46XY, testis with an undeveloped genital tubercle), renal hypoplasia
G040	2875_2902del	Leu959Trpfs*35	*De novo*	–	+	+	+		–	–		–	–	–		–	+	Syndactyly
G012 F*	2941dup	Asp981Glyfs*103	*De novo*	+	–	+	–	+	+	+	–	+	+	+	–	NA		Agnathia, hypoplastic maxillary, absence of oral orifice, bilateral choanal atresia, oligosyndactyly, arthrogryposis, mesomelia bilateral radio-ulnar bowing, absence of tibia and fibula, bilateral renal agenesis, pituitary gland agenesis, adrenal agenesis, uterovaginal aplasia, AVC, CCA, microcephaly
G024 F*	3006_3007insT	Arg1003*	*De novo*	+	+	+	–	+	+	+/–	–	–	+	–	+	NA		Posterior cleft palate, micrognathia, micromelia, oligosyndactyly, club feet, adrenal gland hypoplasia, bilateral renal agenesis, anteposed anus
G013 F*	3040G>T	Glu1014*	*De novo*	+	+	+	+	+	+	+	–	+	+	+	+	NA	+	Bilateral choanal atresia, retrognathia, posterior cleft palate, ear dysplasia, cervical chondroma, syndactyly, adrenal and pituitary gland agenesis, micropenis, unilateral renal agenesis, abnormal aortic arch

Table 2 (Continued)

N°	cDNA alteration (c.)	Predicted protein alteration (p.)	Inheritance	Growth delay/GH deficiency	Insertional/ postaxial PD	Brachytelephalangism/ dactyly	Y-shaped metacarpal/ metatarsal	Hypothalamic hamartoma	Craniofacial anomalies	Anal atresia	Bifid epiglottis	Cardiac anomalies	Renal anomalies	Genital anomalies	Lung dysplasia	Intellectual deficiency	Nail dysplasia	Other findings
G080 F*	3098dup	Ala1034Glyfs*50	Sporadic	+	–	–	+	+	+	+	+	+	+	+	+	NA		Premaxillary agenesis, microretrognathism, arhinencephaly, hygroma colli, intestinal malrotation, micropenis, bilateral renal agenesis, IAC, IVC, adrenal gland agenesis
G104 F*	3324C>G	Tyr1108*	*De novo*	–	+	+	+		+	+	–	+	+	+	+	NA		Hypertelorism, retrognatism, cleft palate, oligosyndactyly, abnormal metacarpals, unilateral renal agenesis, micropenis, IAC
G081	3386_3387del	Phe1129*	*De novo*	–	–	+	–	+	–	+	+	–	–	+		+	+	Limited ankle mobility, syndactyly, hypopituitarism, micropenis, hypospadias, speech delay, gelastic seizures

Abbreviations: AVC, atrioventricular communication; CC, corpus callosum; CCA, corpus callosum agenesis; DSD, developmental sexual disorder; F*, Fetal case; IAC, interauricular communication; IVC, interventricular communication; NA, non applicable; PD, polydactyly. #, Index cases are indicated in bold and related are below.

Table 3 Frequencies of clinical features in GCPS individuals

Features	*Frequency*
Facial anomalies	
Widely spaced eyes	43% (20/47)
Macrocephaly	60% (32/53)
Craniosynostosis	4% (2/55)
Hand anomalies	
Preaxial polydactyly	7% (4/55)
Postaxial polydactyly	45% (25/55)
Broad thumbs	22% (12/55)
Foot anomalies	
Preaxial polydactyly	73% (40/55)
Postaxial polydactyly	20% (11/55)
Syndactyly	64% (34/53)
Cerebral anomalies	
Corpus callosum anomalies	50% (9/18)
Ventricular dilatation	39% (7/18)
Developmental delay	
Severe	11% (5/45)
Mild	20% (9/45)
Birth weight >4000 g	12% (7/55)
Inguinal or umbilical hernia	11% (6/54)

caused by haploinsufficiency of *GLI3* and adjacent genes confirmed by array-CGH with a deletion of 7 and 9 Mb, respectively. Both presented preaxial PD of the feet, developmental delay and ophthalmologic findings (strabismus, cataract). The mutations segregated with the disease in all familial cases. In one apparently sporadic case (G070), the mutation was inherited from a healthy mother with no sign of GCPS.

Pallister–Hall syndrome. We identified heterozygous *GLI3* mutations in 21 patients from 17 families with features of PHS (HH and/or insertional or postaxial PD and/or Y-shaped metacarpal) and all were truncating (12 frameshift and 5 nonsense). Among the 13 sporadic cases, the *de novo* occurrence was confirmed in 13. Three mutations were previously reported in other patients (c.2149C>T, c.3040G>T and c.3386_3387del)[9,18] and 14 mutations were novel. All mutations identified in probands with PHS were 3′ of the DNA-binding domain and predicted the formation of a truncated protein. All were located in the previously described PHS region stretching from aa 667 to 1161, one starting just one amino-acid upstream, which predicts a premature termination codon 27 triplets downstream (P15112). All mutations found in fetuses with severe phenotypes affect a delineated region of the middle third of *GLI3* in the transactivation/CBP-binding region (Figure 1).

Clinical and radiological findings

GCPS

Limb anomalies. Preaxial PD of the feet was the most frequent finding (40/55) (Figures 2a, b and d), but broad halluces were also observed (Figure 2c). Complete preaxial PD of the hands was seen only in 1 case (Figure 2f) and broad thumbs in 12 cases (Figure 2g) with the presence in 2 cases of a delta phalanx or a bifid terminal phalanx on X-rays (Figure 2e). Postaxial PD was observed in 25/55 cases (45%) in feet (20%) or hands (45%). The severity of the PD

Table 4 Frequencies of clinical features in PHS individuals

Clinical features	*Frequency*
Growth delay/GH deficiency	53% (10/19)
Limb anomalies	
Y-shaped metacarpal	83% (15/18
Postaxial polydactyly	48% (10/21)
Insertional polydactyly	48% (10/21)
Preaxial polydactyly	0% (0/21)
Oligodactyly	14% (3/21)
Syndactyly	38% (8/21)
Brachydactyly/brachytelephalangism	52% (13/21)
Mesomelia	19% (4/21)
Nail hypoplasia	69% (9/13)
Overlapping toes	9% (2/21)
Cerebral anomalies	
Hypothalamic hamartoma	100% (12/12)
Corpus callosum anomalies	17% (2/12)
Craniofacial anomalies	
Micro/retro/agnathia	24% (5/21)
Choanal atresia	14% (3/21)
Cleft palate	14% (3/21)
Chondroma	9% (2/21)
Bifid epiglottis	44% (4/9)
Anal anomalies	
Anal imperforation	43% (9/21)
Anteposed anus	5% (1/21)
Cardiac anomalies	
Interauricular communication	9% (2/21)
Interventricular communication	9% (2/21)
Atrioventricular communication	5% (1/21)
Aortic arch anomaly	5% (1/21)
Renal anomalies	41% (7/17)
Genital anomalies	48% (10/21)
Lung dysplasia	50% (4/8)
Developmental delay	21% (3/14)
Seizures	13% (2/15)

extended from a pedunculated postminimus (Figure 2h) to a fully formed supernumerary digit (Figure 2f). Syndactyly present in 64% (34/53) may occur in any limb and varied from partial to complete cutaneous syndactyly of the digits. Metacarpals were not affected except the first metacarpal, sometimes shorter and squatter (Figure 2b).

Craniofacial dysmorphism. In our series, 43% of patients had widely spaced eyes and 60% had macrocephaly. Scaphocephaly and trigonocephaly were noted both in one case.

Cerebral anomalies. A brain MRI was performed in 18 patients. A ventricular dilatation was found in seven cases. Surprisingly, corpus callosum dysgenesis (hypoplasia or agenesis) were not found in patients with large deletion except one but mainly in those bearing a truncating mutation in the C-terminal region of *GLI3* (7/9). In one family (G006), corpus callosum abnormalities were present in all affected individuals. Hypoplastic cerebellum was found in two patients (G005, A018) without molar tooth sign. Among patients with a large deletion encompassing *GLI3*, 5/6 manifested developmental delay and 4 had abnormal brain MRI findings. Apart from these deleted cases, a mild developmental delay (fine motor delay) was observed in nine cases. Among them, seven had a C-terminal mutation, the two others had neurofibromatosis type 1 and prematurity complications.

Other occasional signs. Other less common anomalies in GCPS included umbilical and inguinal hernias (six patients). Birth weight was indicated for 15 patients and macrosomia was noted for 7.

Pallister–Hall syndrome

Limb anomalies. Limb anomalies were present in all 21 PHS individuals of our cohort. The most common feature was postaxial (48%) or insertional PD (48%) (Figures 3a and c). No patient with preaxial PD was recorded. Interestingly, Y-shaped metacarpal/metatarsal was visualized on X-rays in 83% of cases and in all other cases, numeric or morphologic anomalies of metacarpal/metatarsal were noted (Figures 3c and d). Only one patient (P15112) had bilateral and symmetrical Y-shaped metacarpals without PD (Figure 3d). Brachydactyly with brachytelephalangism was observed in at least 52% of PHS cases and nail hypoplasia in 69%.

Syndactyly (38%) and overlapping toes (9%) were frequently reported. Four fetuses with severe phenotypes exhibited mesomelia or micromelia and three of them presented oligodactyly, club feet and arthrogryposis (Figures 3b, g–j).

Neurological findings. A HH was present in 12 patients, all with mutations falling in the 'PHS' domain (Figures 3e and f). In one fetus, neuropathological examination of the hypothalamic region found histological lesions of hamartoma, although a macroscopic mass was not visualized in the infandibular region (G024). Two cases displayed corpus callosum dysgenesis. Most patients had a normal intellectual efficiency, only three were slightly delayed. Seizures were reported in two cases as gelastic epilepsy.

Other findings. IUGR was found in 4/5 fetuses and growth was delayed in 6 patients. Besides, the endocrine manifestations of a HH ranged from isolated growth hormone deficiency (4/13) to panhypopituitarism (1 case); 4/5 fetuses displayed adrenal hypoplasia. Oral anomalies were reported in all prenatal cases: cleft palate in three fetuses, micro/retrognatia in four and unexpectedly, a complete agnathia with absence of oral orifice in one (Figure 3c). Laryngeal examination revealed bifid epiglottis in half cases, always asymptomatic in postnatal cases. Choanal atresia was present in three patients and two displayed cervical or preauricular chondroma. Moreover, imperforate or anteposed anus was present in half PHS cases including all fetuses. Congenital heart defects were diagnosed in six patients: interauricular septal defect in two, interventricular communication in two, atrioventricular septal defect in one and an aortic arch anomaly in one. Renal anomalies were present in 41% ranging from kidney hypoplasia to agenesis and resulting in oligoamnios in prenatal cases with bilateral agenesis (three cases). Genitourinary anomalies including micropenis (nine cases), hypospadias (one case) and uterovaginal aplasia (one case) were present in half of the individuals. A severe developmental sexual disorder was present in a girl with a male caryotype exhibiting an undeveloped genital tubercle. Lung anomalies including abnormal lobulation or hypoplatic lungs were present in four individuals.

In situ hybridization of *GLI3*

GLI3 expression pattern was studied during early human development using *in situ* hybridization on human embryo sections at CSs 14 (day 32), 16 (day 40), 18 (day 44), and 19 (day 47) and at 8.5 weeks of

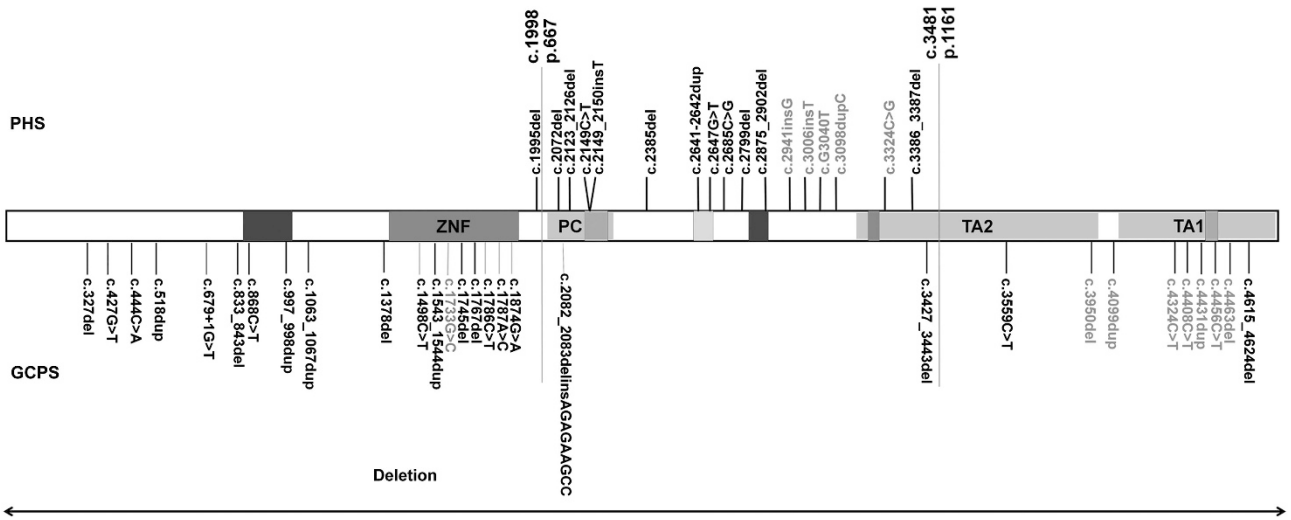

Figure 1 Schematic representation of GLI3 domains and localization of the *GLI3* mutations reported in this study. Red bars at the nucleotides 1998 and 3481 divide the gene into three segments, limiting the PHS region as described elsewhere.[9] The colored boxes within GLI3 represent the seven regions of similarity between human GLI proteins originally defined by Ruppert *et al*.[34] ZNF: zinc-finger domain (aa 462–645), PC: proteolytic cleavage site, TA1 (aa 1376–1580) and TA2 (aa 1044–1322): two independent transactivation domains as described by Kalff-Suske *et al*.[11] Mutations written in red: PHS patients with severe phenotypes; in green: GCPS cases with abnormal corpus callosum; black bars: truncating nonsense and frameshift mutations; purple bar: splice mutation; blue bars: missense mutations. A full color version of this figure is available at the *European Journal of Human Genetics* journal online.

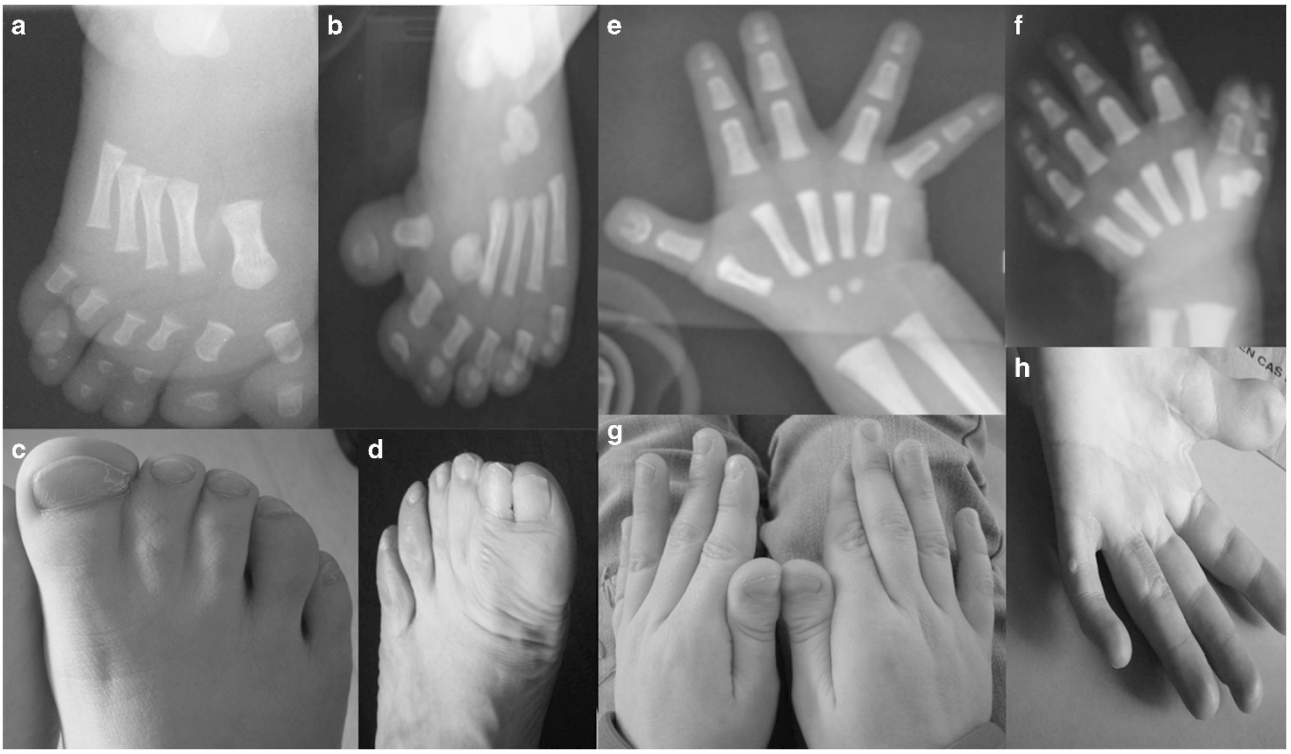

Figure 2 Photographs and radiographies of GCPS cases with identified *GLI3* mutation. (**a**, **b**) Preaxial polysyndactyly in the feet with a broad first metacarpal on X-rays (G076, G14083). (**c**) Broad hallux and syndactyly (G16012, daughter). (**d**) Preaxial polysyndactyly (G16012). (**e**) Bifid terminal phalanx of the thumb (G14083). (**f**) Heptadactyly (preaxial and postaxial PD, G15198). (**g**) Broad thumbs (G16012, daughter). (**h**) Broad thumb and postaxial PD type (**b**) (G16012).

development. At day 32, *GLI3* was strongly expressed in ventral part of the prosencephalon, the mesencephalon and neural tube. *GLI3* expression was also expressed in otic vesicle. At day 40, the expression pattern was observed in pharyngeal archs and was restricted later at day 49 in maxillary and mandible. At the same time, *GLI3* was also expressed in distal limb buds, floor plate of the telencephalon, diencephalon and mesencephalon, neural tube and strongly in kidney (Figure 4).

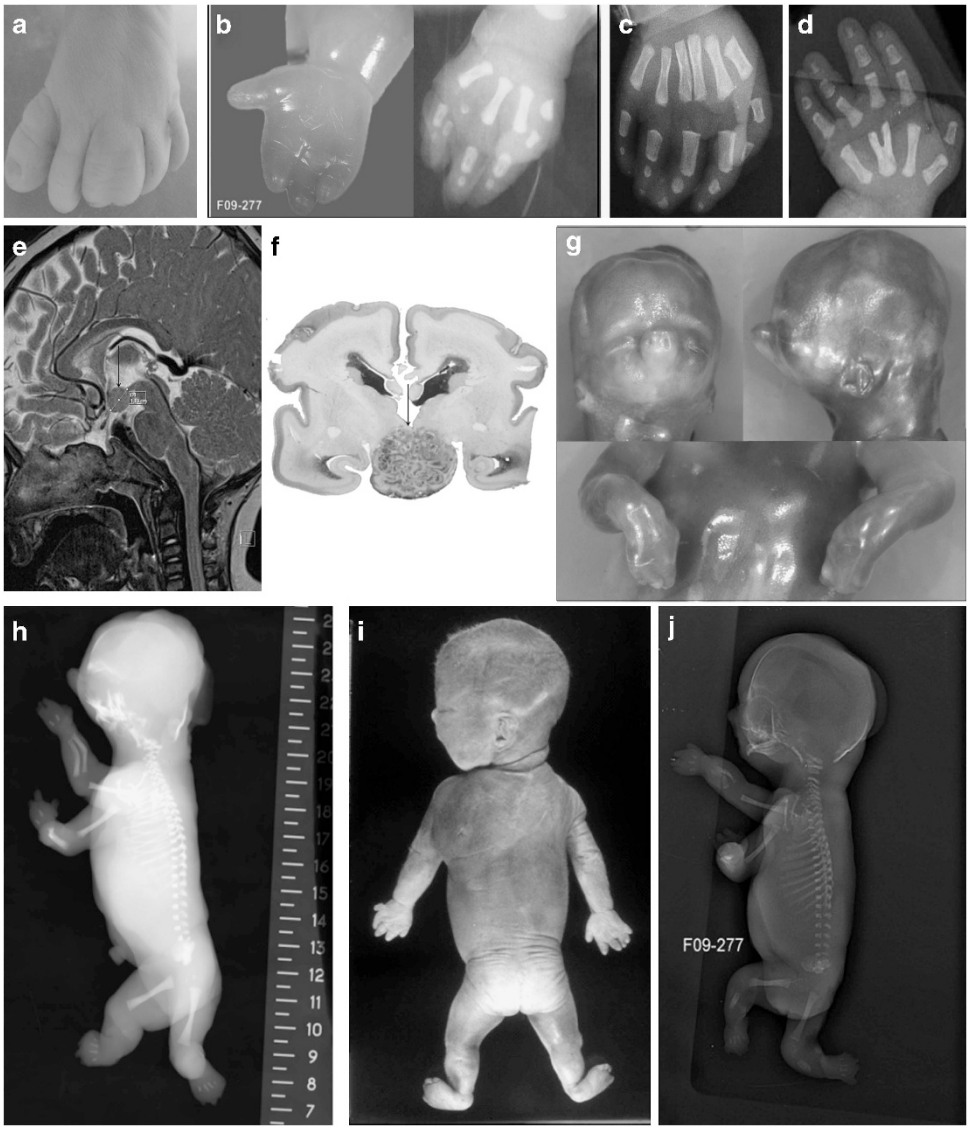

Figure 3 Photographs, radiographies and histological findings of PHS cases with identified *GLI3* mutation. (**a**) Insertional PD and syndactyly (G072). (**b**) Oligodactyly (G080). (**c**) Insertional PD with a supernumerary metacarpal (G072). (**d**) Y-shaped metacarpal without PD (P15112). (**e**) Brain MRI showing a HH (P15112). (**f**) HH on neuropathological examination (G013). (**g**) Agnathia, hypoplastic maxillary, absence of oral orifice and oligosyndactyly (G012). (**h**) X-rays of G012 showing oligosyndactyly of hands and feet, arthrogryposis, mesomelia, bilateral radio-ulnar bowing, absence of tibia and fibula (G012). (**i**) (G024) and (**j**) (G080) showing micrognathia, micromelia, oligosyndactyly and club feet.

DISCUSSION

We present in this report molecular and clinical data of the second largest series of patients with *GLI3* mutations. Molecular results of our series support previous genotype–phenotype correlations, showing that exonic deletions, missense mutations, as well as truncating variants localized outside the middle third of the *GLI3* gene result in GCPS, while truncating mutations in the middle third result in PHS. Two truncating mutations in patients with preaxial PD mapped within the PHS region. Previous known exceptions to these correlations were described, for example, the recurrent c.2374C>T associated with a typical GCPS phenotype.[9,11,19,20] These exceptions may be explained by a variable contribution of nonsense-mediated decay[12] or by the formation of unstable proteins with a very short half-life, effective nulls.

Only 10 GCPS probands of our series did fulfill all criteria suggested by Biesecker *et al*[6] namely preaxial PD, cutaneous syndactyly, widely spaced eyes and macrocephaly. Craniofacial features were absent or very subtle in 17 patients. Craniosynostosis was found in only two patients confirming the low-frequency association with GCPS.[21] Along the same line, the diagnosis of PHS was confirmed in four individuals despite the absence of PD, whereas the clinical diagnostic criteria for PHS classically require the presence of insertional PD and a HH in the proband.[22]

Interestingly, corpus callosum anomalies were found in nine patients, including seven patients with a truncating mutation located in the third end of *GLI3*. In the two previous series reported by Johnston *et al*,[9,10] four GCPS patients with corpus callosum dysgenesis were also carrying a *GLI3* truncating mutation lying in the C-terminal domain of the protein further confirming our finding that corpus callosum dysgenesis is fully part of GCPS spectrum and is mainly caused by terminal truncating mutations. Overlapping features with acrocallosal syndrome (ACLS, MIM# 200990) associating callosal

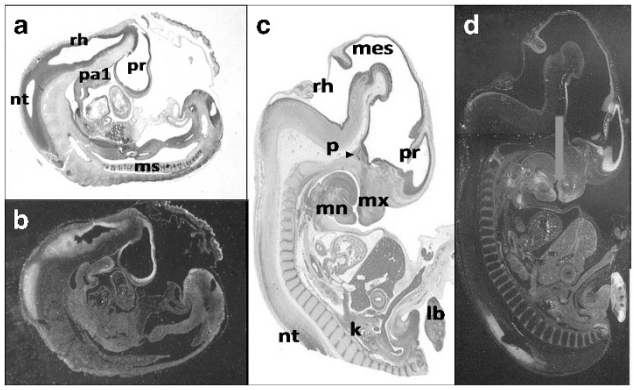

Figure 4 *In situ* hybridization of *GLI3* during human development. (**a**, **b**) CS 15; (**c**, **d**) CS 19. (**b**, **d**) Slides hybridized with an antisense *GLI3* probe. (**a**, **c**) Adjacent slides respectively to (**b**) and (**d**) stained with HES. In addition to the expression in central nervous system (prosencephalon (pr), rhombencephalon (rh) neural tube (nt)), limb bud (lb), pituitary gland (p, arrowhead) and kidney (K), a signal was observed in human pharyngeal arches (pa1) then in mandible (mn) and maxillary (mx) (red arrows). A full color version of this figure is available at the *European Journal of Human Genetics* journal online.

dysgenesis, hypertelorism, intellectual disability and PD[23] are explained by an impaired GLI3 processing in patients with *KIF7* mutations.[24] Facial dysmorphism, as well as vermis dysgenesis with brainstem anomalies (molar tooth sign), strongly indicated the diagnosis of ACLS. Conversely, two *GLI3* mutated cases with corpus callosum dysgenesis have been reported as ACLS[25,26] and a third similar patient has been reported by Johnston *et al.*[10] All three mutations were missense and clustered in the same region between aa 903 and 934 suggesting a potential severe phenotype associated with alterations of this region. Whatever, mutation analysis in both genes is therefore essential as the distinction between these two syndromes is of obvious significance for genetic counseling considering the difference in heredity and neurodevelopmental outcome and patients with a *GLI3* mutation may be diagnosed as GCPS.

Interestingly, macrosomia was observed in at least 13% of GCPS cases in our series. Macrosomia and PD are also observed in Simpson–Golabi–Behmel syndrome type 1 (MIM# 312870), a X-linked mental retardation syndrome ascribed to *Glypican3 (GPC3)* mutation, which was suspected in family G068, with two brothers displaying macrosomia and PD at birth. The frameshift *GLI3* mutation was inherited from their asymptomatic father carrying a somatic mosaic mutation. Along the same way, we identified a mosaic large deletion in a GCPS patient with developmental delay (G059). To our knowledge, only one instance of *GLI3* germline mosaicism has been already described in two PHS sibs,[18] which is therefore a rare event.

Cerebral MRI may be useful to detect HH that was found in all PHS individuals of our series. Abnormal metacarpals in particular Y-shaped metacapals appear to be a more significant criterion than insertional PD. At least three PHS patients without PD were already reported.[10,18,27] All of them presented fused or hypoplastic metacarpal. The mouse model for PHS $Gli3^{\Delta 699/\Delta 699}$ displays abnormal metacarpal morphology with PD or oligodactyly at a lower frequency.[28] Central poly/syndactyly and Y-shaped metacarpals are extremely uncommon in other syndromes. Although associated to a good neurodevelopmental outcome, PHS displays a wide range of severity varying from mild to lethal phenotypes depending on the severity of malformations present in the individuals, in particular bilateral kidney agenesis, craniofacial features (agnathia, absence of oral orifice, cleft palate or premaxillary agenesis in one case), heart defects and/or reductional limb defects. Interestingly, skeletal dysplasia with ulna bowing, tibia and fibula hypoplasia was already reported in other cases described as PHS,[10,29,30] further suggesting that acromesomelic limb shortening with radio-ulnar bowing, tibial and fibular hypolasia/agenesis are part of the phenotypic spectrum of PHS. Interestingly, all mutations found in severe fetal phenotypes of our series were clustered in the middle third of the gene, between c.2941 and c.3324. To assess whether the severe craniofacial features observed (agnathia, absence of oral orifice) were a direct effect of the *GLI3* mutation, we undertook the expression analysis of *GLI3* during human development. Indeed, in addition to the early expression of *GLI3* in pharyngeal and then later in mandible and maxillary, *GLI3* was highly expressed in all target tissues of the disease.

Besides PHS cases, Y-shaped metacarpal is also observed in oro-facio-digital syndrome type VI (OFD VI; MIM# 277170).[31] Overlap of PHS with OFD has been previously discussed as oral anomalies (oral frenula, hamartoma, cleft palate) and/or skeletal dysplasia are often associated to *GLI3* mutations.[10] Avila *et al*[32] screened eight patients with OFD associated with midline abnormalities but no mutation was found. They suggested that *GLI3* should be screened in patients with OFD only when associated to one of the pathognomonic sign of PHS (HH, mesoaxial PD, bifid epiglottis and IA).

In one case (G013), the association of IUGR, PD, bilateral renal agenesis and anal anteposition without macroscopic HH first led to the suspicion of Smith–Lemli–Opitz (SLO; MIM# 270400). After exclusion of a cholesterol biosynthesis defect, a *GLI3* screening identified a *de novo* frameshift mutation in exon 15. Retrospective analysis of the brain identified microscopic changes suggestive of hamartoma. This observation underlines the phenotypic overlap of PHS and SLO that was suggested previously,[22] both disorders associating IUGR, PD and possible renal agenesis but IA, insertional PD and HH are exceptional in SLO.[33]

CONCLUSION

Here we report on clinical and molecular data of a large series of 76 individuals from 55 families carrying a heterozygous *GLI3* mutation or rearrangement, 55 GCPS and 21 PHS, 41 being novel mutations. Our results render more precisely the genotype–phenotype correlation of *GLI3* mutations proposed by Johnston *et al*, and further highlight the clinical overlap between GCPS and ACS and between PHS, SLO and OFD. Interestingly, our series including fetal cases enlarge the phenotypic spectrum of PHS to severe craniofacial and reductional limb defects, emphasize on the possible lethality of PHS with a clustering of truncating mutation in a subdomain of the 'PHS' *GLI3* domain. In addition, we add CCA among frequent signs of GCPS with a strong genotype–phenotype correlation of corpus callosum dysgenesis with truncating C terminal mutations, and macrosomia as a new clinical feature of GCPS.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

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SIFT: http://blocks.fhcrc.org/sift/SIFT.html
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