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Molecular and Clinical Analyses of Greig Cephalopolysyndactyly and Pallister-Hall Syndromes: Robust Phenotype Prediction from the Type and Position of *GLI3* Mutations

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Mutations in the *GLI3* zinc-finger transcription factor gene cause Greig cephalopolysyndactyly syndrome (GCPS) and Pallister-Hall syndrome (PHS), which are variable but distinct clinical entities. We hypothesized that *GLI3* mutations that predict a truncated functional repressor protein cause PHS and that functional haploinsufficiency of *GLI3* causes GCPS. To test these hypotheses, we screened patients with PHS and GCPS for *GLI3* mutations. The patient group consisted of 135 individuals: 89 patients with GCPS and 46 patients with PHS. We detected 47 pathological mutations (among 60 probands); when these were combined with previously published mutations, two genotype-phenotype correlations were evident. First, GCPS was caused by many types of alterations, including translocations, large deletions, exonic deletions and duplications, small in-frame deletions, and missense, frameshift/nonsense, and splicing mutations. In contrast, PHS was caused only by frameshift/nonsense and splicing mutations. Second, among the frameshift/nonsense mutations, there was a clear genotype-phenotype correlation. Mutations in the first third of the gene (from open reading frame [ORF] nucleotides [nt] 1–1997) caused GCPS, and mutations in the second third of the gene (from ORF nt 1998–3481) caused primarily PHS. Surprisingly, there were 12 mutations in patients with GCPS in the 3′ third of the gene (after ORF nt 3481), and no patients with PHS had mutations in this region. These results demonstrate a robust correlation of genotype and phenotype for *GLI3* mutations and strongly support the hypothesis that these two allelic disorders have distinct modes of pathogenesis.

Introduction

Mutations in the *GLI3* zinc-finger transcription factor on chromosome 7p14.1 cause the Pallister-Hall syndrome (PHS [MIM 146510]) (Kang et al. 1997*b*) and the Greig cephalopolysyndactyly syndrome (GCPS [MIM 175700]) (Vortkamp et al. 1991), both of which are inherited in an autosomal dominant pattern. Although both disorders manifest postaxial polydactyly, PHS and GCPS are distinct clinical entities with numerous nonoverlapping features (Biesecker et al. 1996; Gorlin et al. 2001). Clinical features that distinguish the disorders include hypothalamic hamartoma, bifid epiglottis, and insertional polydactyly in PHS and preaxial polydactyly and hypertelorism in GCPS. Three types of apparently isolated polydactyly—PAP-A, PAP-A/B, and PPD-IV—are also caused by *GLI3* mutations (Radhakrishna et al. 1997, 1999), and a *GLI3* mutation was reported in a child with features of GCPS and acrocallosal syndrome (Elson et al. 2002). Therefore, the clinical phenotypes caused by mutations in *GLI3* are diverse and complex.

GLI3 acts as both a transcriptional activator and a transcriptional repressor of downstream targets in the sonic hedgehog (SHH) pathway during development (Ruiz i Altaba 1999). In the presence of SHH, full-length GLI3 up-regulates target genes, whereas, in the absence

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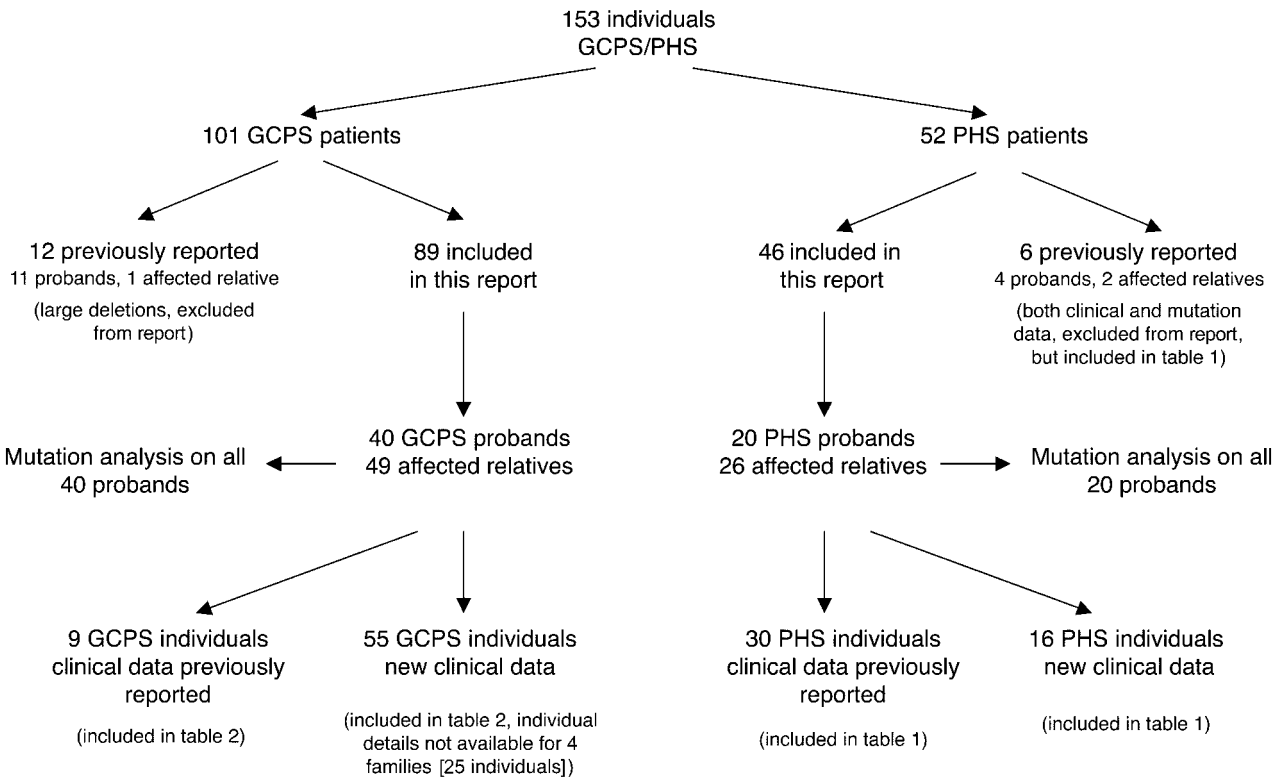

Figure 1 Flow chart showing numbers of patients included in each part of the study. Notice that, for completeness, data for all patients except those with large deletions are included in tables 1 and 2. (Some of the data have been published elsewhere, as noted.)

of SHH, GLI3 is cleaved to produce a repressor that down-regulates target genes. Initial genotype-phenotype studies led to the hypothesis that GCPS results from functional haploinsufficiency, a hypothesis that is supported by the fact that deletions or translocations affecting *GLI3* have been described only for individuals with GCPS (Wagner et al. 1990; Vortkamp et al. 1991) and not for individuals with PHS. Additionally, point mutations in the DNA-binding domain were identified in individuals with GCPS (Kalff-Suske et al. 1999). In contrast, the first two mutations identified in families with PHS were truncating mutations just 3′ of the region that encodes the DNA-binding domain (Kang et al. 1997*b*). Truncated proteins from these alleles were predicted to act as constitutive repressors. As the number of reported mutations in *GLI3* increased, the proposed genotype-phenotype correlation was called into question. Indeed, the phrase "GLI3 morphopathies" was coined to describe the phenotypes caused by *GLI3* mutations, because the apparent lack of genotype-phenotype correlation precluded phenotypic classification (Radhakrishna et al. 1999). To resolve this, we analyzed a large cohort of probands with features of GCPS and/or PHS. Here, we report the clinical and molecular analyses of 135 patients from 60 families and correlate the mutations with the clinical manifestations.

Methods

Patients

This study was reviewed and approved by a National Institutes of Health (NIH) institutional review board (IRB). The *GLI3* project originally included 153 individuals with PHS or GCPS. Twelve patients with GCPS were found to have large deletions of *GLI3* and have been reported elsewhere (Johnston et al. 2003). In addition, six patients with PHS have had both clinical and mutation results reported elsewhere (Killoran et al. 2000; Galasso et al. 2001; Turner et al. 2003; Ng et al. 2004); the remaining 135 subjects (60 probands and 75 affected relatives) are reported in the present study (fig. 1). Clinical manifestations in individuals from seven families (PH01, PH02 [Kang et al. 1997*a*], PH06 [Low et al. 1995], PH17 [Verloes et al. 1995], M19 [Zucchini et al. 1998], GB01 [Baraitser et al. 1983], and GB05 [Ridler et al. 1977]) have been described elsewhere; two other probands (from families PH22 [Stroh et al. 1999] and

PH42 [Haynes and Bagwell 2003]) were the subjects of case reports after they were enrolled in the present study but have been included in the "Results" section because significant additional clinical information was collected. Four additional families (GB02, GB03, GB04, and GB07) have been excluded from the "Results" section of the present study because adequate phenotypic information about them was not available. The novel clinical data therefore include 71 patients (49 probands and 22 affected relatives) (individual details given in tables 1 and 2). Of the 49 families, 38 were clinically evaluated at the NIH. This evaluation included a history, physical examination, limb radiographs, and cranial imaging. In accordance with NIH IRB guidelines, young patients might not have had cranial imaging studies under sedation in the absence of clinical indications. Most patients with PHS were analyzed for bifid epiglottis (Ondrey et al. 2000). Insertional polydactyly is defined as the presence of a floating metacarpal or metatarsal between the second and fifth (or most posterior) metacarpals or metatarsals or the fusion of metacarpals or metatarsals other than the first or most posterior. Preaxial polydactyly was evaluated on the basis of whether phalanges were fully or partially duplicated. The assessment of wide or broad thumbs and big toes was a subjective clinical assessment. The clinical diagnostic criteria for PHS require the presence of insertional polydactyly and a hypothalamic hamartoma in the proband (Biesecker et al. 1996). Family members are considered affected if they have isolated hamartoma or polydactyly and if they are a first-degree relative of an affected individual. Specific clinical diagnostic criteria have not been set for GCPS (Biesecker 2001); however, suggested criteria include preaxial polydactyly in at least one limb or abnormally wide big toes or thumbs, syndactyly, macrocephaly, and hypertelorism. For the present study, we set relaxed GCPS eligibility criteria of preaxial polydactyly and the presence of at least one additional feature (syndactyly, macrocephaly, hypertelorism, or postaxial polydactyly). Family members were considered affected if they had polydactyly or abnormally wide big toes or thumbs and were a first-degree relative of an affected individual. Statistical comparisons were performed by Fisher's exact test (InStat), unless otherwise specified.

DNA Isolation

DNA was isolated from whole blood by use of the salting-out method (Gentra), per the manufacturer's instructions.

Sequence Analysis

Sequencing of the *GLI3* coding exons was performed with BigDye terminator cycle sequencing kit v.3 and either the ABI 377 or ABI 3100, per the manufacturer's protocol (Applied Biosystems Group). Sequence data were compared with the published *GLI3* sequence (GenBank accession number NM_000168.2; University of California–Santa Cruz (UCSC) genome assembly, July 2003 freeze) (UCSC Genome Bioinformatics) by use of Sequencher 4.12 software.

DHPLC Analysis

For some probands, a screening of exons 3–12 and the last third of exon 15 was performed using denaturing high-performance liquid chromatography (DHPLC) (Transgenomic). Primers used for PCR amplification are shown in table 3, as are gradient temperatures, gradient durations, and initial starting concentrations of proprietary buffer B (Transgenomic). All gradients were run using the rapid-gradient option, and the increase in buffer B was 5% per min for all gradients. All amplicons that produced peak patterns that differed from those of wild-type controls were sequenced.

Controls for missense variations were also performed by DHPLC. Controls were run under a single gradient condition capable of identifying the nucleotide change of interest (table 4). Eighty ethnically matched control individuals (160 chromosomes) were analyzed for each alteration.

qPCR Analysis

Quantitative PCR (qPCR) analysis of the *GLI3*-coding exons was performed with Platinum SYBR Green qPCR SuperMix UDG kit (Invitrogen) and ABI PRISM 7000 (Applied Biosystems Group), per the manufacturers' instructions. Endogenous control amplicons included an STS on chromosome 7 and an amplicon from the deoxynucleotide carrier (*DNC*) gene on chromosome 17q25.3 (Iacobazzi et al. 2001). A single amplicon from each coding exon of *GLI3* was assayed. Amplification primers for each *GLI3* exon and the controls are listed in table 5. Copy number was determined using a combination of standard curve and comparative C_T method of analysis.

RT-PCR Analysis

RNA was isolated from lymphoblasts by use of affinity chromatography (RNAeasy and Qiashredders [Qiagen]). A combined RT-PCR reaction was performed using Qiagen's One Step RT-PCR kit, per the manufacturer's instructions, with the addition of proprietary solution Q. Products were cloned into the TOPO cloning vector (Invitrogen) and were sequenced using BigDye terminator cycle sequencing kit v.3 and ABI 377 (Applied Biosystems Group).

Table 1

Clinical Findings and Mutation Status for Patients with PHS

Individual[a]	DNA Alteration (Exon)	Predicted Protein Alteration	Inheritance[b]	Finding or Symptom[c]								Additional Findings[c]
				Hypothalamic Hamartoma	Insertional Polydactyly	Postaxial Polydactyly	Bifid Epiglottis	Nail Hypoplasia	Dental Hypoplasia	DD/MR[d]	SZ/HD[e]	
PH34	c.1998_2001del4 (13)	p.P668_T669delinsLfsX24	F	+	HB	−	+	+	−	−	SZ, HD	
PH51	c.2032delG (13)	p.D678fsX15	F	+	FB	HR	+	+	−	−	−	Imperforate anus
PH22[f]	c.2062G→T (13)	p.E688X	S	+	HL	HR	+	+	+	−	−	Genital hypoplasia
PH53	c.2110C→T (14)	p.Q704X	S	+	HR	HL	+	+	−	DD	−	Precocious puberty
PH41	c.2139delC (14)	p.C713fsX713	S	+	HB	HR	NA	+	+	DD	SZ	Genital hypoplasia, growth hormone deficiency
M19[f]	c.2146C→T (14)	p.Q716X	S	+	+	+	NA	NA	NA	NA	NA	Vaginal atresia, urogenital sinus, bifid uvula
PH36	c.2149C→T (14)	p.Q717X	S	+	HL	FR, HR	+	+	+	DD, MR	SZ	Imperforate anus
PH06-1[f]	c.2157delC (14)	p.I720SfsX13	F	+	HR	−	NA	+	NA	−	−	
PH06-2				+	HB	−	NA	NA	NA	−	−	
PH01-1[f]	c.2172_2173insC (14)	p.N725QfsX13	F	+	HB	−	+	NA	−	−	HD	Hypothyroidism
PH01-8				−	HB	−	NA	+	−	−	−	Bilateral hypodactyly on feet, RF preaxia polydactylyl
PH01-10				+	HB, FR	−	NA	+	NA	−	HD	Loss of right kidney function
PH01-13				+	HB, FR	−	+	NA	NA	−	HD	Dental crowding
M05[g]	c.2188_2207del20 (14)	p.L730X	S	+	HR	−	NA	+	NA	NA	NA	Imperforate anus, small kidney, hypoplastic iliac bone
PH31-1[g]	c.2197_2198delAC (14)	p.T733RfsX4	F	+	−	HR, FL	NA	NA	NA	NA	NA	
PH31-2				+	HB, FR	−	NA	+	NA	NA	NA	Oral frenula
PH42-1[f]	c.2346_2356del11 (14)	p.R782_V786delinsSfsX15	F	+	HB	HB	+	+	NA	DD	SZ	Imperforate anus, cortisol and thyroid deficiency
PH42-2				+	HB, FL	−	NA	NA	NA	NA	HD	
PH21	c.2351_2355del5 (14)	p.K784_Q785delinsSfsX15	S	+	HB	−	+	−	+	DD	HD	Genital hypoplasia, growth hormone deficiency
PH02[f]	c.2431+1G→A	IVS14	F	+	++	++	++	NA	NA	NA	++HD	
PH49	c.2483delC (15)	p.P828RfsX14	S	+	HL	HR	+	+	+	−	SZ, HD	

PH61	c.2567C→A (15)	p.S856X	S	+	HL	HL	+	+	−	DD	SZ	Imperforate anus, hydro-metrocolpos, short limbs, hearing loss, vesico ureteric reflux left kidney, low tone
PH23	c.2620delC (15)	p.R874fAsX16	S	+	HL	−	+	+	+	DD	SZ, HD	Bilateral hearing loss
PH15	c.2628delC (15)	p.S877AfsX13	S	+	FB, HB	−	+	+	+	DD	SZ, HD	Genital hypoplasia, growth hormone deficiency, unilateral hearing loss
PH33[g]	c.2770_2771ins72 (15)	p.A924delins(12)X	S	+	+	−	+	+	NA	DD	−	
PH39	c.2799C→G (15)	p.Y933X	S	+	FL	−	NA	−	+	NA	NA	Imperforate anus, tracheal stenosis, anal/vaginal fistula, syndactyly hand (digits 4 and 5)
PH20	c.3324C→G (15)	p.Y1108X	S	+	FB, HB	−	+	−	−	NA	NA	Imperforate anus, laryngeal cleft, one functional kidney
PH43-3[g]	c.3386_3387delT (15)	p.F1129X	F	+	HR	HL, FL	+	+	−	MR	SZ	Imperforate anus
PH43-4				+	HB	−	+	+	−	MR	SZ	Hypothyroidism, imperforate anus
PH32	c.3456G→T (15)	p.E1152X	S	+	HR	HB, FL	+	+	+	MR	SZ, HD	Imperforate anus, hearing loss, LF preaxial polydactyly
PH17[f]	No mutation		S	+	HB	−	+	+	−	Severe MR	−	Growth hormone deficiency, precocious puberty
Totals[h]				29/30	29/30	12/30	19/19	21/24	9/19	13/23	17/24	

[a] The first two digits specify family number; when more than one individual is reported in a family, the family number is followed by an individual number. Family PH02 had a total of 22 affected relatives; individual data not presented.

[b] F = familial; S = sporadic.

[c] B = bilateral; F = foot; H = hand; L = left; R = right; a plus sign (+) = demonstrated in individual; a double plus sign (++) = demonstrated in multiple family members; a minus sign (−) = not demonstrated in one or more individuals; NA = not assessed or data not available in one or more individuals.

[d] DD = developmental delay; MR = mental retardation.

[e] HD = headache; SZ = seizures.

[f] Clinical analysis published elsewhere (Low et al. 1995; Verloes et al. 1995; Kang et al. 1997*a*; Zucchini et al. 1998; Stroh et al. 1999; Haynes and Bagwell 2003).

[g] Clinical and mutation analysis published elsewhere (Killoran et al. 2000; Galasso et al. 2001; Turner et al. 2003; Ng et al. 2004).

[h] Totals do not include family PH22. All totals are shown as the number of individuals with finding/total number of individuals assessed.

Table 2

Clinical Findings and Mutation Status in Patients with GCPS

Individual[a]	DNA Alteration (Exon)	Predicted Protein Alteration	Inheritance[b]	Finding or Symptom[c]						MRI Findings[c,d]
				Insertional Polydactyly	Postaxial Polydactyly	Preaxial Polydactyly	Syndactyly	Macrocephaly	Hypertelorism	
GB04 (3)	c.540_547del8 (5)	p.N181_T183delinsCfsX15	F	–	NA	FB	FB	NA	NA	
G48	c.658delC (5)	p.R220VfsX3	S	–	HB	FB1, HB2	FB, HB	+	+	
G16-1	c.679+2_679+15del14	IVS5	F	–	–	FB3	FB, HB	+	–	Normal
G16-2				–	–	FB2	FB, HR	+	–	NA
G16-4				–	–	FB1	FB, HB	–	–	Normal
G60-1	c.827-3C→G	IVS6	F	–	HR	HB3	FB	+	+	NA
G60-3				–	HB	FL1, FR3	–	+	NA	Normal
G12-2	c.868C→T (7)	p.R290X	F	–	–	FB1	–	+	+	Normal
G12-3				–	–	FB1, HL2	–	–	–	Normal
G12-4				–	HB	FB1	–	–	NA	Normal
G18-1	c.868C→T (7)	p.R290X	F	–	HB	FB3	FB, HB	–	NA	NA
G18-2				–	–	FB1	FB, HB	–	NA	NA
G30-1	c.868C→T (7)	p.R290X	F	–	–	FB3	–	NA	NA	Normal
G30-2				–	–	FB1	–	NA	NA	Normal
G30-3				–	HB	FB1	HB	–	NA	Normal
G39-1	c.868C→T (7)	p.R290X	F	–	HB	FB1	FB, HB	+	NA	NA
G39-2				–	HB	FB1	FB, HB	–	NA	NA
G39-3				–	HB	FB1	FB, HR	NA	+	NA
G15	c.1048_1049insT (8)	p.Y350LfsX62	S	–	HB	FB1	HB	–	+	
GB01 (6)[e]	c.1074delC (8)	p.H358QfsX7	F	–	HB, FB	FB	FB, HB	+	+	
G04	c.1497+1G→C	IVS10	S	–	HB	FB1	FB	–	–	Normal
G14-2	c.1497+1G→A	IVS10	F	–	HR	FB1, HR2	–	–	–	Normal
G14-3				–	–	FB1	–	NA	NA	NA
G66-1	c.1497+1G→T	IVS10	F	–	HB	FB2	FB, HB	–	–	NA
G66-2				–	–	FB1	FB	+	–	Normal
GB05 (3)[e]	c.1617_1633del17 (11)	p.R539_P545delinsSfsX7	F	NA	H	FB	FB, HB	–	–	
G47	c.1789C→T (12)	p.Q597X	S	–	HR	FB1	–	+	NA	
G74	c.1880_1881delAT (13)	p.H627RfsX48	S	–	HB	FB1	–	+	+	
G44-2	c.2374C→T (14)	p.R792X	F	–	–	FB2	–	–	–	Normal
G44-3				–	HR	FB1	–	+	NA	NA
G44-4				–	HB	FB2	FB	+	–	Normal
GB02 (8)	c.2374C→T (14)	p.R792X	F	–	HB	FB	FB, HB	+	–	

GB03 (4)	c.2374C→T (14)	p.R792X	F	−	−	FB	HB	NA	NA	
G08	c.4119_4123 delins7 (15)	p.P1374_S1375delinsAfsX2	S	FL	HL	FB2, HB3	FB, HB	−	+	Agenesis of corpus callosum
G05	c.4402_4403insG (15)	p.L1469AfsX10	S	−	FB, HB	FR1	HL	+	+	
M23	c.4427delA (15)	p.S1477fsX11	S	−	HB, FR	+	−	−	NA	
G82-1	c.4564delG (15)	p.A1522PfsX2	F	−	FB, HB	HB2	−	+	+	Thin corpus callosum
G82-2				−	FB, HB	FL3, HB2	−	+	+	Normal
G82-4				−	FB, HB	FR3	FB	+	+	Normal
G06	c.4677_4678insC (15)	p.R1560RfsX38	S	−	FR, HB	FB1	−	+	+	Partial agenesis of corpus callosum
G32-1	c.1446C→G (10)	p.C482W	F	−	HR	FB1, HB2	−	−	−	Normal
G32-2				−	−	FB1	−	+	−	NA
G25-1	c.1874G→A (13)	p.R625Q	F	−	−	FB2	HB	−	−	Normal
G25-2				−	−	FB1	HL	+	−	Normal
G79	c.1873C→T (13)	p.R625W	S	−	HB	FB	HL, 2–3 toe	NA	+	NA
G19	del. ex. 2–15		S	−	−	FB1	HB	−	+	
G46-1	dup. ex. 8–12		F	−	−	FB1	FB	+	−	NA
G46-2				−	HL	FB1, HL2	FB	−	+	Normal
G63-1	del. ex. 3–4		F	−	−	FB1	FB, HB	+	−	NA
G63-2				−	−	FB1, HB3	FB	−	+	Arachnoid cyst
G63-3				−	−	FB1, HB3	FB	+	+	Normal
G67	del. ex. 2		S	−	HL	FB	−	+	NA	Normal
G33	No mutation		S	−	HB	HR	FB, HB	−	−	
G52	No mutation		S	−	HB	FB1	FB	−	−	Normal
G53	No mutation		S	−	−	FB1	−	+	NA	Normal
G62	No mutation		S	−	−	FL3, FR2	FB, HB bony	−	NA	Normal
G65	No mutation		S	−	HL	FB1	HB	−	−	
G68	No mutation		S	−	−	FB2	−	+	NA	Normal CT
G69	No mutation		S	−	FB, HB	FR2	−	NA	−	
Ph63	No mutation		S	−	FB	HB	−	NA	NA	Normal
GB07 (10)	No mutation[f]		F	−	HB	FB	FB, HB	+	+	CNS glioma
Totals[g]				1/55	33/55	55/55	32/55	25/48	17/37	

[a] The first two digits specify family number; when more than one individual is reported in a family, the family number is followed by an individual number. Individual data are not available for families GB01, GB02, GB03, GB04, GB05, and GB07; number in parentheses denotes the number of affected individuals.

[b] F = familial; S = sporadic.

[c] B = bilateral; F = foot; H = hand; L = left; R = right; 1 = complete; 2 = partial; 3 = abnormally wide; a plus sign (+) = demonstrated in individual; a minus sign (−) = not demonstrated in one or more individuals; NA = not assessed or data not available in one or more individuals.

[d] MRI = magnetic resonance imaging.

[e] Clinical analysis published elsewhere (Ridler et al. 1977; Baraitser et al. 1983).

[f] Exonic deletion/duplication analysis was not performed, because DNA was unavailable.

[g] Totals do not include families GB01, GB02, GB03, GB04, GB05, and GB07, since the clinical data for these families are available only in aggregate form; that is, no data on individuals are available. All totals are shown as the number of individuals with finding/total number of individuals assessed.

Table 3

Primer Sets and Amplicon Lengths for Mutation Detection with DHPLC

The table is available in its entirety in the online edition of *The American Journal of Human Genetics*.

Results

This article describes novel data (clinical or molecular or both) for 135 patients from 60 families, including 20 probands and 26 affected relatives with PHS and 40 probands and 49 affected relatives with GCPS. This includes novel clinical data for 71 patients and novel molecular data for 60 probands. Clinical and mutation data are presented in tables 1 and 2 for patients with PHS and GCPS, respectively.

Mutation Detection

Causative mutations were identified in 19 probands with PHS from 20 families and in 28 probands with GCPS from 40 families. Mutations are listed in tables 1 and 2 and are shown diagrammatically in figure 2*B*. Mutation analyses included both sequencing of coding exons and intron/exon junctions and, when additional DNA was available, exonic copy-number analysis by use of qPCR to exclude subgenic deletions in individuals when sequencing did not reveal alterations.

PHS Mutations

The diagnostic yield for PHS was 95%, since only 1 of 20 individuals who met the PHS diagnostic criteria did not have an identifiable mutation in *GLI3*. Mutations in patients with PHS included frameshift and nonsense mutations in exons 13, 14, and 15 and one splice-site mutation in intron 14 in one family (PH02). Analyses of RNA from a hybrid cell line containing this splice mutation showed production of three splice products. Sequence analyses of these products showed two splice products that predict frameshifts at aa 702 and aa 786 (p.T702AfsX14 and p.V786AfsX14, respectively). The other splice product predicts an in-frame deletion of 75 aa (p.G736_N810del). All mutations identified in probands with PHS were 3′ of the DNA-binding domain and predicted the formation of a truncated protein.

GCPS Mutations

Mutations were identified in 28 (70%) of the 40 probands with GCPS. Alterations in patients with GCPS included splice-site, frameshift, and nonsense mutations and exonic deletions and duplications. Sequence analyses revealed 24 causative mutations with only the recurrent p.R792X mutation—identified in three separate families (G44, GB02, and GB03)—falling within the PHS mutation area (see the "Discussion" section). This mutation has been reported in three other families with GCPS (Kalff-Suske et al. 1999; Debeer et al. 2003). Distinct missense alterations were also identified in 3 (8%) of 40 probands. The first missense alteration, p.C482W in family G32, affects a conserved cysteine in the C2H2 zinc-finger domain and likely abrogates DNA binding. The second and third missense alterations, p.R625Q in family G25 and p.R625W in family G79, affect a highly conserved arginine in the C2H2 zinc-finger domain; p.R625W has been reported elsewhere (Debeer et al. 2003). Missense alterations p.C482W and p.R625Q segregated with the disease and were not identified in 160 ethnically matched control chromosomes. Missense alteration p.R625W was not present in either parent; paternity was confirmed.

When a mutation or missense alteration was not detected by sequence analysis and additional DNA was available, qPCR was performed to detect exonic deletions or duplications. Three deletions and one duplication were identified among four probands with GCPS. In patient G19, previous STRP analysis had suggested that the region surrounding *GLI3* on chromosome 7 was deleted. All STRP markers tested were homozygous/hemizygous in the individual, but parental samples were not available to confirm a deletion. The qPCR result confirmed that the entire *GLI3* gene is deleted in the individual. In patient G63, qPCR analysis showed a deletion of exons 3 and 4, and RT-PCR analysis showed exon 2 spliced directly to exon 5, which resulted in a large out-of-frame deletion (p.E42GfsX58). The qPCR result for patient G67 predicted a deletion of exon 2. In patient G46, qPCR predicted a duplication of exons 8–12, a minimum of 48.8 kb.

Discussion

GLI3 mutations have been associated with several phenotypes, including GCPS, PHS, isolated polydactyly, and acrocallosal syndrome. Initially, large genomic deletions and translocations were found in patients with GCPS and led to a mechanistic model of haploinsufficiency (Wagner et al. 1990; Vortkamp et al. 1991). Subsequently, point mutations were discovered in individuals with GCPS (Wild et al. 1997). These could plausibly fit a haploinsufficiency model, since many were within or upstream of the DNA-binding domain and were predicted to cause loss of function (Wild et al. 1997). In contrast

Table 4

Primer Sets and Amplicon Lengths for Analysis of Missense Alteration Controls with DHPLC

The table is available in its entirety in the online edition of *The American Journal of Human Genetics*.

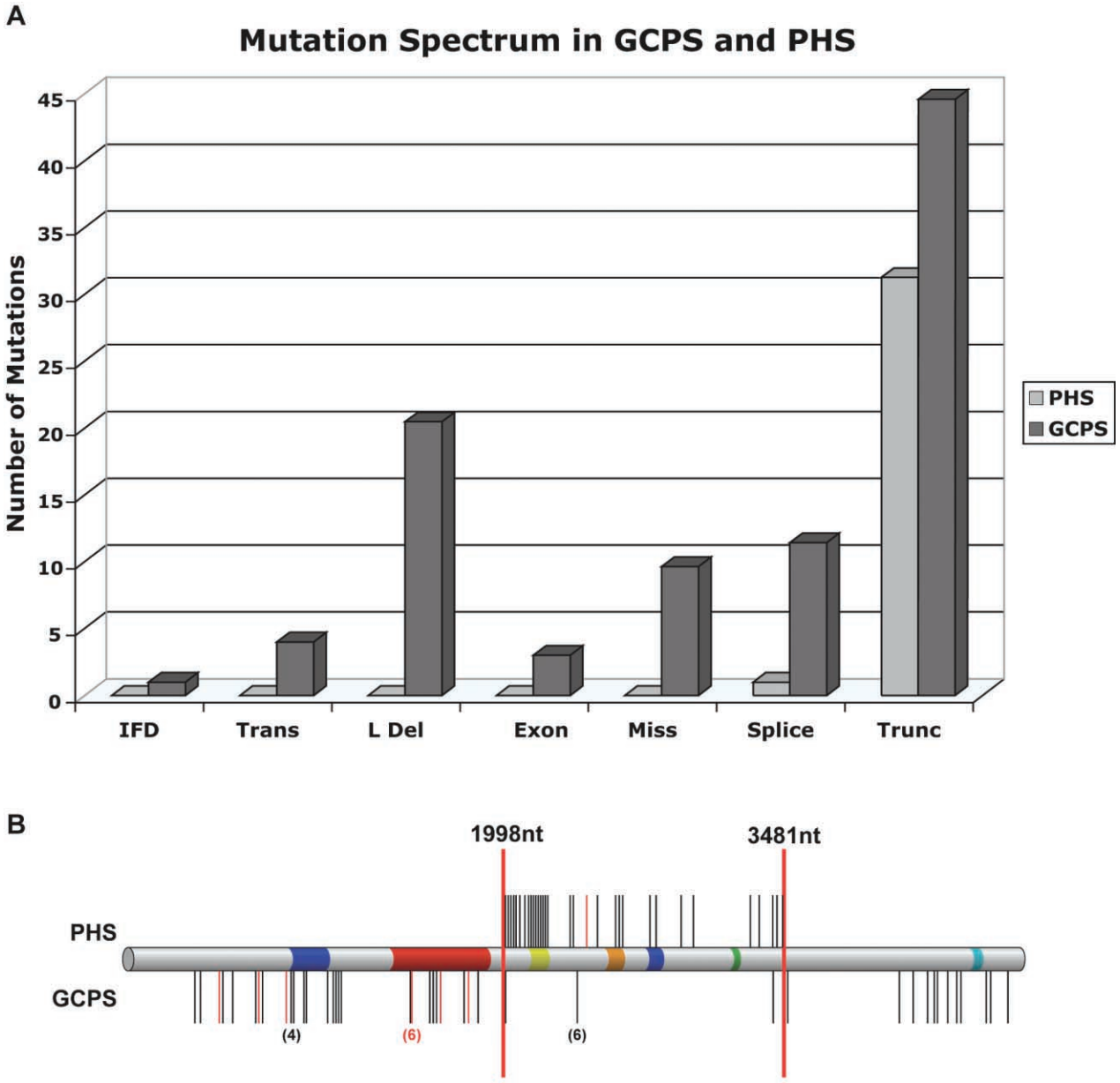

Figure 2 Type and distribution of *GLI3* mutations described for patients with PHS and GCPS. *A,* Mutation spectrum. No mutations of the following types have been described for patients with PHS: small in-frame deletions (IFD), translocations (Trans), large deletions (L Del), exonic deletions or duplications (Exon), and missense mutations (Miss). The correlation of mutation type and phenotype is statistically significant ($P < .0001$ [Fisher's 2 × 2]) when the classes of mutations were dichotomized into frameshift and nonsense (Trunc) versus all other types and when tested against phenotype (PHS vs. GCPS). *B,* Diagram of the position within the gene of known nonsense, frameshift, and splice-site mutations. Some of the closely spaced mutations have been adjusted for increased visual clarity. Splice-site mutations are shown in red. Black numbers indicate identical mutations, and red numbers indicate multiple mutations in the same splice donor. Data include published mutations (Bianchi et al. 1981; Tommerup and Nielsen 1983; Marks et al. 1985; Pelz et al. 1986; Wagner et al. 1990; Pettigrew et al. 1991; Vortkamp et al. 1991; Kang et al. 1997*b;* Radhakrishna et al. 1997, 1999; Wild et al. 1997; Williams et al. 1997; Kalff-Suske et al. 1999, 2000*a,* 2000*b,* 2004; Friez and Stevenson 2000; Killoran et al. 2000; Galasso et al. 2001; Kroisel et al. 2001; Elson et al. 2002; Debeer et al. 2003; Driess et al. 2003; Freese et al. 2003; Johnston et al. 2003; Kremer et al. 2003; Turner et al. 2003; Ng et al. 2004) and the mutations identified in the present study. The colored bars on the protein show the conserved domains of GLI3 as defined elsewhere (Ruppert et al. 1990).

Table 5

Primer Sets and Amplicon Lengths for Mutation Detection with qPCR

The table is available in its entirety in the online edition of *The American Journal of Human Genetics*.

to GCPS, mutations reported for individuals with PHS consistently predicted truncation of the protein C-terminal of the DNA-binding domain. The model was expanded to include a constitutive repressor as the mechanism for PHS (Biesecker 1997), which has subsequently been validated by in vitro experimental data (Wang et al. 2000) and a mouse model of PHS (Bose et al. 2002). However, as the *GLI3* mutation spectrum grew, it was suggested that clear genotype-phenotype correlations were not apparent, and the phrase "GLI3 morphopathies" was proposed (Radhakrishna et al. 1999) to describe allelic *GLI3* phenotypes without clear phenotypic boundaries. To resolve these two opposing views, we analyzed additional patients and compiled mutation data from the literature.

To understand genotype-phenotype correlations, it is critical to have a clear understanding of the phenotypes. The diagnostic criteria for PHS require the presence of (1) a hypothalamic hamartoma and insertional polydactyly in the proband or (2) isolated hamartoma or polydactyly in a relative of the proband with PHS (Biesecker et al. 1996). We analyzed 20 probands (and 26 additional affected relatives) who met the PHS diagnostic criteria. Of those 20 probands, 19 had a mutation 3′ of the *GLI3* zinc-finger domain. The spectrum of mutations found in patients with PHS was limited to nonsense, frameshift, and a single splice mutation (which generated mostly frameshifted transcripts). The diagnostic yield with the use of the PHS clinical criteria is 95% (for finding a mutation of this type in this region of *GLI3*). These results are surprisingly robust and suggest that, when the clinical criteria are met, clinical geneticists can rapidly and confidently make a diagnosis of PHS and can institute appropriate medical care, which may be life saving (Biesecker 2003). Mutation analysis is supportive and useful for many reasons but is not necessary for initiation of this care.

In contrast to the situation for PHS, defined diagnostic criteria do not yet exist for GCPS. This is not surprising, since GCPS comprises relatively few features, which are relatively nonspecific and are found in numerous other disorders. For example, preaxial polydactyly is recognized as a manifestation of at least 25 syndromes (Stevenson et al. 1993), and hypertelorism is recognized as a symptom of at least 68 syndromes (Jones 1997). The situation for PHS is very different; hypothalamic hamartoma is recognized as a symptom of only four syndromes and insertional polydactyly of only six. It is important to emphasize that the clinical diagnostic challenge for GCPS is to distinguish it from disorders other than PHS, since PHS and GCPS are readily distinguished from each other. The suggested diagnostic criteria for GCPS include preaxial polydactyly in at least one limb or abnormally wide big toes or thumbs, syndactyly, macrocephaly, and hypertelorism. For the present study, we set relaxed GCPS eligibility criteria of preaxial polydactyly and the presence of at least one additional feature (syndactyly, macrocephaly, hypertelorism, or postaxial polydactyly).

The cohort described here (with the use of the relaxed criteria) included 89 patients (40 probands and 49 affected relatives) with GCPS. Of the five individuals who fulfilled the strict diagnostic criteria for GCPS, all five (100%) of the individuals had mutations in *GLI3*. In the complete cohort of 40 probands, mutations were identified in a total of 28 (70%), and additional missense alterations were revealed in 3 (8%). Although the diagnostic yield for mutation detection in patients who fulfilled the strict GCPS clinical criteria was 100%, the difference in the two diagnostic yields was not statistically significant. In addition, the relaxed clinical criteria allowed many more cases to be screened, so the absolute yield was higher. An intriguing observation is that hypertelorism is uncommon among the relaxed-criteria group who were not found to have a mutation in *GLI3*. Further study of this subgroup is necessary to determine the clinical and biological significance of this apparent finding.

We conclude that many patients who present with features in the GCPS spectrum may not have manifestations amenable to phenotypic diagnosis because of variable severity and the nonspecific clinical features of GCPS. The presentation of a patient with some (but not all) features of GCPS may require clinicians to have a high level of clinical suspicion and to follow up with molecular diagnostics. We suggest that these relaxed clinical criteria would be most useful in selecting patients for molecular analysis instead of in making a clinical diagnosis in the absence of molecular data. Fortunately, GCPS is not associated with life-threatening complications (as is PHS), so withholding diagnosis until molecular confirmation can be made is an appropriate strategy.

The spectrum of *GLI3* mutations in patients with GCPS is large, and there are no efficient algorithms to detect all of these mutations. We have suggested (Johnston et al. 2003) that patients with suspected GCPS should have a standard giemsa-banded karyotype (to exclude the presence of translocations). If the result is normal, it should be followed by *GLI3* FISH. If the FISH results are also normal, it should be followed by *GLI3*-mutation scanning by use of sequence analysis and, if necessary, qPCR or another method for detection

of exonic copy number. Further research is necessary to make this algorithm more sensitive and less expensive, since it would currently cost at least U.S. $3,000 for these four tests (Johnston et al. 2003).

When the mutation data from the existing literature (Bianchi et al. 1981; Tommerup and Nielsen 1983; Marks et al. 1985; Pelz et al. 1986; Wagner et al. 1990; Pettigrew et al. 1991; Vortkamp et al. 1991; Wild et al. 1997; Williams et al. 1997; Kalff-Suske et al. 1999, 2000*a,* 2000*b,* 2004; Radhakrishna et al. 1999; Friez and Stevenson 2000; Killoran et al. 2000; Galasso et al. 2001; Kroisel et al. 2001; Elson et al. 2002; Debeer et al. 2003; Driess et al. 2003; Freese et al. 2003; Johnston et al. 2003; Kremer et al. 2003; Turner et al. 2003; Ng et al. 2004) and the present study are compiled, a clear genotype-phenotype correlation emerges. We approached the question of genotype-phenotype correlation on two levels: first, attempting to correlate distinct types of mutations with the phenotypes and, second, correlating the positions of mutations within the gene for the classes of mutations (frameshift, nonsense, and splicing) that are present in both disorders. When all mutation types are included, a correlation is demonstrated. Patients with GCPS have translocations, large deletions (i.e., detectable by FISH assay with BAC probes), exonic deletions and duplications, small in-frame deletions, and missense, splicing, frameshift, and nonsense mutations (a total of 92 mutations [fig. 2*A*]). In striking contrast, only nonsense, frameshift, and a single splice-site mutation were found in 32 patients with PHS. To test for significance, we dichotomized mutation types into two groups, truncating and nontruncating; these two groups were significantly ($P < .0001$) correlated with phenotype (PHS and GCPS). We conclude that these data refute prior assertions of a lack of correlation of *GLI3* genotype and phenotype (Radhakrishna et al. 1999).

However, additional insights can be gained by analysis of the classes of mutations—frameshift, nonsense, and splicing—that are shared by both phenotypes. We and others (Kalff-Suske et al. 1999) have shown that mutations 3′ of the zinc-finger domain are present in patients with GCPS, and it was unclear how these mutations differed from those identified in patients with PHS. When all frameshift, nonsense, and splicing mutations are combined, mutations causing GCPS and PHS fall in distinct regions of *GLI3* (fig. 2*B*). PHS is typically caused by mutations in the middle third of the gene, whereas GCPS is caused by mutations outside that region. Although we had previously hypothesized (Biesecker 1997) that all frameshift or nonsense mutations 3′ of the DNA-binding domain would cause PHS or PAP-A, it is now clear that there are patients with mutations in the far 3′ end of the gene and that all of them have GCPS. There are several potential mechanisms that could explain a GCPS phenotype with these mutations. The presence of a truncated protein C-terminal of aa 1161 may make the protein subject to ubiquitination or other modes of protein degradation, the protein may be mislocalized to an organelle or other subcellular structure that leads to loss of function, or these truncations may trigger nonsense-mediated mRNA decay. This will be difficult to test, because GLI3 is present at very low levels in adult cells and because it is technically challenging to engineer numerous mutations into model organisms (i.e., mouse) to test these hypotheses.

The genotype-phenotype correlation is not perfect. Intriguingly, there are now six independent families (including three families reported here) with GCPS and the p.R792X nonsense mutation, which is in the PHS mutation region. The three families with the p.R792X mutation reported here clearly had GCPS, and the brief clinical data from the prior reports of patients with the p.R792X mutation suggest that they most likely have GCPS as well (Kalff-Suske et al. 1999; Debeer et al. 2003). This causation of GCPS by this mutation is difficult to explain, but the mutation may generate a protein that is targeted for degradation or an mRNA that is subjected to nonsense-mediated mRNA decay. Preliminary data argue against mRNA decay, since the mutant and wild-type message levels appear similar in a lymphoblast line from a patient with p.R792X (from family G44) (data not shown). Two other mutations, p.R667X and p.L1146RfsX95, overlap known PHS mutations. The p.R667X mutation is adjacent to the 5′ border of the PHS domain (aa 666), and p.L1146RfsX95 predicts a protein that is highly charged at the carboxyl end, which may affect its function. Additional research is needed to determine if these mutations conflict with or correspond to our model of GLI3 pathogenesis. The splice-site mutation found in family PH02 with PHS is interesting, since the data reported here suggest that the mutation is consistent with our model. This splice-site mutation generates several cDNA products, including an in-frame product with a 75-aa deletion (predicting p.G736_N810del) and two products that predict frameshifts at aa 702 and aa 786 (p.T702AfsX14 and p.V786AfsX14). We conclude that this mutant allele generates only a portion of the transcripts that are repressors (since two of three sequenced splice variants have frameshifts) and a portion of transcripts with an in-frame deletion. This is consistent with the phenotype in this family (a large family with mild PHS) and with the model of truncated repressor alleles causing PHS.

The PHS and GCPS phenotypes breed true and correlate strongly with mutation type and position. A *GLI3* deletion, translocation, small in-frame deletion, exonic deletion/duplication, or missense mutation causes GCPS (as found in 37 patients with GCPS and no patients with PHS). A *GLI3* nonsense, frameshift, or splice-site mu-

tation causes GCPS if it occurs 5′ of nt 1998 or 3′ of nt 3481 (as found in 47 patients with GCPS and no patients with PHS) and is correlated with PHS if it occurs between those nucleotides (as found in 8 patients with GCPS and 32 patients with PHS). The data show a clear correlation of genotype and phenotype on two levels (mutation type with phenotype and mutation position with phenotype for truncating mutations). The data refute prior assertions of a lack of correlation and show how these data can be useful in clinical practice and in refining our models of the physiology and pathophysiology of GLI3 action.

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Electronic-Database Information

The accession number and URLs for data presented herein are as follows:

GenBank, http://www.ncbi.nlm.nih.gov/Genbank/ (for *GLI3* sequence [accession number NM_000168.2])
Online Mendelian Inheritance in Man (OMIM), http://www.ncbi.nlm.gov/Omim/ (for PHS and GCPS)
UCSC Genome Bioinformatics, http://www.genome.ucsc.edu/

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