# New insights into genotype-phenotype correlation for GLI3 mutations 

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## ARTICLE

# New insights into genotype-phenotype correlation for GLI3 mutations 

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#### Abstract

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.?
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). ${ }^{8}$

[^0]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 $5 \mu$ m-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. $1874 \mathrm{G}>\mathrm{A}, \mathrm{c} .4463 \mathrm{del}$ and $\mathrm{c} .444 \mathrm{C}>\mathrm{A}$ 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 aminoacid 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. $868 \mathrm{C}>\mathrm{T}$ and $\mathrm{c} .1874 \mathrm{G}>\mathrm{A}$ ) 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

| $N^{\circ}$ (\#) | cDNA alteration (c.) | Predicted protein alteration (p.) | Inheritance | Postaxial $P D$ | $\begin{gathered} \text { Preaxial } \\ P D \end{gathered}$ |  | $\begin{aligned} & \text { Synd- } \\ & \text { actyly } \end{aligned}$ | Macrocephaly | Widely spaced eyes | $\begin{gathered} \text { MRI } \\ \text { Findings } \end{gathered}$ | Developmental delay | Additional findings |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
| G029 | 327del | Phe109Leufs*50 | F | - | FB |  | + | - | - | - | - | Precocious puberty, scaphocephaly |
| G070 | $427 \mathrm{G}>\mathrm{T}$ | Glu143* | F | HB | FL |  | - | + |  |  | - |  |
| Mother | $427 \mathrm{G}>\mathrm{T}$ | Glu143* | F | - | - |  | - | - | - |  | - |  |
| G118 | $444 C>A$ | Tyr148* | F | - | FB | BT | + | + |  |  | - |  |
| G13684 | $444 \mathrm{C}>\mathrm{A}$ | Tyr148* | F | - | FB |  | + | + | + |  | - |  |
| Brother | $444 C>A$ | Tyr148* | F | - | FL |  | + | - | + |  | - |  |
| Mother | $444 \mathrm{C}>\mathrm{A}$ | Tyr148* | F | - | FB |  | + | + |  |  | - |  |
| G099 | 518dup | \|le174Hisfs*2 | F | - | FB |  | + | + | - |  | - |  |
| G048 | $679+1 G>T$ | Splice | De novo | HB | FB, HB |  |  |  |  | VD |  | Delta phalanx |
| G15198 | 833_843del | Arg278Thrs* 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, $B W=4150$ |
| G033 | 1378del | Val461Serfs*41 | F | - | FB | BT | + | + | + |  | - |  |
| G076 | $1498 \mathrm{C}>\mathrm{T}$ | His500Tyr | F | - | FB |  | + | + | - | + | + | Cerebral prematurity sequelae |
| Mother | $1498 \mathrm{C}>\mathrm{T}$ | His500Tyr | F | - | FB |  | + | - | - |  | - |  |
| Brother | $1498 C>T$ | His500Tyr | F | HB | FB |  | + | - | - |  | - |  |
| G068 | 1543_1544dup | Arg516Alafs*20 | F | - | FB |  | + | - | - |  | - | Delta metacarpal, $\mathrm{BW}=4880$ |
| Brother | 1543_1544dup | Arg516Alafs*20 | F | - | FB |  | + | - | - |  | - | $B W=4740$ |
| Father | [ =/1543_1544dup] | [ = /Arg516Alafs*20] | F | - | - |  | - | - | - |  | - |  |
| A018 | $1733 \mathrm{G}>\mathrm{C}$ | Cys578Ser | S | HB | FB | BT | + | + | + | $\begin{aligned} & \mathrm{CCH}, \\ & \mathrm{VD} \end{aligned}$ |  | Hypoplastic cerebellum, microretrognathism |
| G094 | 1745del | Gly $582 \mathrm{Valfs} * 47$ | 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 | $1786 \mathrm{C}>$ T | His596Tyr | F | HB | FB |  | - | - | + |  |  | Macrosomia |
| Father | $1786 \mathrm{C}>\mathrm{T}$ | His596Tyr | F | - | - | BH | - | - | - |  | - |  |
| Sister | $1786 \mathrm{C}>\mathrm{T}$ | His596Tyr | F | - | FB |  | + | - | + |  |  |  |
| G056 | $1787 \mathrm{~A}>\mathrm{C}$ | His596Pro | F | - | FB |  | + | + | + |  | + | Neurofibromatosis type 1 |
| Father | $1787 \mathrm{~A}>\mathrm{C}$ | His596Pro | F | - | FB |  | + | + |  |  | - |  |
| G026 | $1874 G>A$ | Arg625GIn | S | - | FB, HB |  | + | - | - |  | - | Brachydactyly, delta phalanx |
| G14331 | 1874 > $\times$ A | Arg625GIn | De novo | - | FB | BT, BH | + | + | + |  |  | Brachydactyly, speech delay |
| G111 | 2082_2083delinsAGAGAAGCC | Val695Glufs*45 | F | HB | FB |  | - | - | - |  | - |  |
| G063 | 3427_3443del | Phel 143Alafs*98 | F | - | FB |  | - | - | + | vD | + | Speech delay, exomphalos |
| G003 | $3559 \mathrm{C}>\mathrm{T}$ | GIn1187* | F | HB | - |  | - | + | + |  | MD |  |
| Brother | $3559 C>T$ | GIn1187* | F | HB, FB | - | BT | - | + | + |  | - |  |
| Mother | $35590>$ T | GIn1187* | F | HB | - |  | - | - |  |  | - |  |

Table 1 (Continued)

| $N^{*}$ (\#) | cDNA alteration (c.) | Predicted protein alteration (p.) | Inheritance | Postaxial <br> PD | $\begin{gathered} \text { Preaxial } \\ P D \end{gathered}$ |  | $\begin{aligned} & \text { Synd- } \\ & \text { actyly } \end{aligned}$ | Macrocephaly | Widely spaced eyes | $\begin{gathered} \text { MRI } \\ \text { Findings } \end{gathered}$ | Developmental delay | Additional findings |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
| G051 | 3950del | Pro1317GInfs*102 | F | HB | - |  | - | + | - | pCCA | Mild |  |
| Mother | 3950del | Pro1317GInfs*102 | F | HB | FB |  | - | + | - |  |  |  |
| A019 F* | 4099dup | Ala1367Glyfs*45 | S | HB, FB | - |  | + | - | + | pCCA |  |  |
| G004 | $4324 \mathrm{C}>$ T | GIn1442* | S | HB, FB | - | BT | - | + | + | pCCA | + | Umbilical hernia, anterior anus |
| G002 | $4408 \mathrm{C}>\mathrm{T}$ | GIn1470* | F | HB, FB | - |  | + | + | + | $\begin{aligned} & \mathrm{CCH}, \\ & \mathrm{VD} \end{aligned}$ | - | Laryngomalacia |
| G006 | 4431dup | Glu1478* | F | HB, FB | - |  | - | + | + | CCH |  | $B W=4440$ |
| A017 | $4456 \mathrm{C}>$ T | GIn1486* | 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.(35674000_37280000)_ (46 116000_46598000)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, $B W=4280$ |  |
| 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]/ <br> 7p14.1(GLI3x2)[44] | S | - | FB |  | - | - | - | - | DD | Trigonocephaly, macrosomia |  |


Table 2 Clinical features and GLI3 molecular results in PHS cases

| $N$ | $\begin{gathered} \text { cDNA } \\ \text { alteration (c.) } \end{gathered}$ | Predicted protein alteration (p.) | Inheritance | $\begin{aligned} & \text { Growth } \\ & \text { delay/GH } \\ & \text { deficiency } \end{aligned}$ | Insertional/ <br> postaxial <br> PD | Brachytelephalangism/ dactyly |  | Hypothalamic <br> hamartoma | Craniofacial anomalies | Anal <br> atresia | $\begin{aligned} & \text { Bifida } \\ & \text { epiglottis } \end{aligned}$ | Cardiac anomalies | Renal anomalies | Genital anomalies | $\begin{aligned} & \text { Lung } \\ & \text { s dysplasia } \end{aligned}$ | Intellectual deficiency | $\begin{gathered} \text { Nail } \\ \text { dysplasia } \end{gathered}$ | Other findings |
| :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: | :---: |
| P15112 | 1995del | Gly666Alafs*27 | De novo | - | - | + | + | + | + | + | + | - | - | - | - | - | + | Overlapping toes, preauricular tag |
| G097 | 2072del | Gln691Argfs*2 | De novo | + | + |  | + | + | - | - |  | - | - | + | - | - |  | Micropenis, thin CC |
| G085 | 2123_2126del | Gly708Vals*24 | Familial | - | + |  | + |  | - | - |  | - |  | - |  | - | + |  |
| Father | Gly708Valfs*24 | Gly708Vals*24 |  | + | + |  | + |  | - | - |  | - |  | + |  | - |  | Micropenis |
| Aunt | Gly708Valfs*24 | Gly708Vals*24 |  |  | + | + |  |  | - | - |  | - |  | - |  | - | + |  |
| Grand-mother | Gly708Valfs*24 | Gly708Vals*24 |  |  | + |  |  |  | - | - |  | - |  | - |  | - | + |  |
| G121 | 2149_2150ins | Glin717Leufs*21 | ? | - | + |  | + |  | - | - |  | - | - | - |  | - | - |  |
| G001 | $2149 C>T$ | GIn717* | De novo | + | + | + | + | + | - | + |  | - | + | + |  | - |  | Sacrococcygeal teratoma, conical teeth, cryptorchidism, micropenis, syndactyly, unilateral renal agenesis, fine motor delay |
| G083 | 2385 del | 11 7966s* ${ }^{\text {d }}$ | Familial | - | + |  | + |  | - | - |  | - | - | - |  | - | - |  |
| Father | 2385 del | 11 796fs* 13 |  | - | + |  | + |  | - | - |  | - | - | - |  | - | - |  |
| G082 | 2641_2642dup | Glin81Hisfs*10 | De novo | + | + | + | + | + |  | + |  | + | - | + |  | + | - | Micropenis, choanal atresia, IVC, fine DD |
| G044 | $2647 \mathrm{G} \times \mathrm{T}$ | Glu883* | Familial | + | + | + | + | + | - | - | - | - | - | - |  | - | + | Scoliosis, dental malposition |
| G072 F* | $2685 \mathrm{C}>\mathrm{G}$ | 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 | Asp981Glysf* ${ }^{*} 103$ | De novo | + | - | + | - | + | + | + | - | + | + | + | - | NA |  | Agnathia, hypoplastic maxil- <br> lary, absence of oral orifice, bilateral choanal atresia, oligosyndactyly, arthrogryposis, mesomelia bilateral radioulnar 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* | $3040 \mathrm{G} \times \mathrm{T}$ | 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 <br> delay/GH <br> deficiency | Insertional/ postaxial PD | Brachytelephalangism/ dactyly | $\gamma$-shaped metacarpal/ metatarsal | Hypothalamic hamartoma | Craniofacial anomalies | Anal <br> atresia | Bifid <br> epiglottis | Cardiac <br> anomalies | Renal anomalies | Genital <br> anomalies | Lung <br> 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* | $3324 \mathrm{C}>\mathrm{G}$ | Tyr1108* | De novo | - | + | + | + |  | + | + | - | + | + | + | + | NA |  | Hypertelorism, retrognatism, cleft palate, oligosyndactyly, abnormal metacarpals, unilateral renal agenesis, micropenis, IAC |
| G081 | 3386_3387del | Phel129* | De novo | - | - | + | - | + | - | + | + | - | - | + |  | + | + | Limited ankle mobility, syndactyly, hypopituitarism, micropenis, hypospadias, speech delay, gelastic seizures |

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 | $7 \%(4 / 55)$ |
| Preaxial polydactyly | $45 \%(25 / 55)$ |
| Postaxial polydactyly | $22 \%(12 / 55)$ |
| Broad thumbs |  |
| Foot anomalies | $73 \%(40 / 55)$ |
| Preaxial polydactyly | $20 \%(11 / 55)$ |
| Postaxial polydactyly | $64 \%(34 / 53)$ |
| Syndactyly |  |
| Cerebral anomalies | $50 \%(9 / 18)$ |
| Corpus callosum anomalies | $39 \%(7 / 18)$ |
| Ventricular dilatation |  |
| Developmental delay | $11 \%(5 / 45)$ |
| Severe | $20 \%(9 / 45)$ |
| Mild | $12 \%(7 / 55)$ |
| Birth weight > 4000g | $11 \%(6 / 54)$ |
| Inguinal or umbilical hernia |  |

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 $3^{\prime}$ 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 <br> 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 |  |
| Anal anomalies | $44 \%(4 / 9)$ |
| Anal imperforation |  |
| Anteposed anus | $43 \%(2 / 15)$ |
| Cardiac anomalies | $43 \%(9 / 21)$ |
| Interauricular communication | $5 \%(1 / 21)$ |
| 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 |  |
|  |  |

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 3 e 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 a/. ${ }^{11}$ 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 (GO72). (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 (GO13). (g) Agnathia, hypoplastic maxillary, absence of oral orifice and oligosyndactyly (GO12). (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. $2374 \mathrm{C}>\mathrm{T}$ 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 GL/3 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 $\mathrm{PD}^{23}$ 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 ${ }^{\text {4699/4699 }}$ 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.

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