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Novel *GLI2* mutations identified in patients with Combined Pituitary Hormone Deficiency (CPHD): Evidence for a pathogenic effect by functional characterization

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Summary

Context: The Gli-family of zinc-finger transcription factors regulates the Sonic Hedgehog (Shh) signalling pathway that plays a key role in early pituitary and ventral forebrain development. Heterozygous *GLI2* loss of function mutations in humans have been reported in holoprosencephaly (HPE), HPE-like phenotypes associated with pituitary anomalies and combined pituitary hormone deficiency with or without other extra-pituitary findings.

Objective: The aim of this study was the search for *GLI2* mutations in a cohort of Italian CPHD patients and the assessment of a pathogenic role for the identified variants through in vitro studies.

Patients: One hundred forty-five unrelated CPHD patients diagnosed with or without extra-pituitary manifestations were recruited from different Italian centres.

Methods: The *GLI2* mutation screening was carried out through direct sequencing of all the 13 exons and intron-exon boundaries. Luciferase reporter assays were performed to evaluate the role of the detected missense variants.

Results: Five different novel heterozygous non-synonymous *GLI2* variants were identified in five patients. The mutations were three missense (p.Pro386Leu, p.Tyr575His, p.Ala593Val), one frameshift (p.Val1111Glyfs*19) and one nonsense (p.Arg1226X). The latter two mutants are likely pathogenic since they lead to a truncated protein. The in vitro functional study of the plasmids bearing two of the three missense variants (namely p.Tyr575His and p.Ala593Val) revealed a significant reduction in transcriptional activity.

Conclusion: In conclusion, the analysis of *GLI2* in individuals with CPHD led to the identification of five variations with a likely negative impact on the GLI2 protein, confirming that *GLI2* is an important causative gene in CPHD. The functional in vitro study analysis performed on the missense variations were useful to strengthen the hypothesis of pathogenicity.

KEYWORDS
CPHD, *GLI2* gene

1 | INTRODUCTION

The *GLI2* (glioma-associated oncogene homolog 2) protein is a transcription factor, member of the vertebrate GLI-Kruppel family that mediates the Hedgehog (HH) signalling.[1] The HH are secreted proteins involved in the embryonic development, stem cell biology and tissue homoeostasis.[2] In particular, the Sonic HH (SHH) ligand activates the HH signalling required for ventral patterning and the expansion of the median forebrain[3-5] and GLI2 represents the primary downstream activator of the HH pathway indispensable to initiate transcription of target genes of the SHH pathway.

The full-length GLI2 protein consists of 1586 amino acids (168 kDa) and contains a central zinc finger DNA-binding domain which is flanked by an amino-terminal (N-terminal) repressor domain and a carboxyl-terminal (C-terminal) transactivation domain. GLI2 has been identified in at least four other isoforms encoded by splice variants, known as α (133 kDa), β (131 kDa), γ (88 kDa) and δ (86 kDa).[6,7] GLI2 acts as transcriptional activator and repressor depending on the isoform and cellular context. Previous studies reported that the full-length GLI2 acts as a weak transcriptional activator, whereas the removal of the N-terminal repressor domain as in the GLI2α variant showed a 30-fold higher activity compared to the full-length protein *in vitro*.[6,8,9]

The *Gli2* knockout mice showed severe skeletal abnormalities including absence of vertebral body and intervertebral disc, truncated mandibles with absent incisors, shortened limbs and sternum, missing tympanic ring bones of the inner ear and severe cleft palate malformations.[9] Moreover, inactivation of *Gli2* resulted in defects of the hindbrain with hypoplastic anterior pituitary and complete loss of posterior pituitary.[10,11] These defects have been attributed to the loss of expression of *Gli2* target genes *Bmp4* and *Fgf8*.[10]

Heterozygous *GLI2* loss of function mutations in humans were initially reported in holoprosencephaly (HPE), a condition characterized by incomplete or failed forebrain separation, or in HPE-like phenotypes with pituitary anomalies and postaxial polydactyly.[6,12] Since then, several *GLI2* sequence variants were reported in patients with HPE characterized by a wide spectrum of phenotypes including craniofacial abnormalities, branchial arch anomalies, polydactyly that may or may not include pituitary defect.[13-16] Subsequently, Franca et al[17,18] reported frameshift or nonsense *GLI2* mutations and a considerable frequency of non-synonymous *GLI2* variants in patients with congenital hypopituitarism without HPE. Most of these patients presented with CPHD and an ectopic posterior pituitary lobe. Since then several individuals with mutations in *GLI2* were reported with typical pituitary anomalies, polydactyly and subtle facial features rather than HPE.[19]

Although several patients have been reported carrying *GLI2* mutations, the effect of most of these variants remains unclear except for those that introduce premature stop codon.[19] To date, the evaluation of the functional significance of missense *GLI2* mutations has been performed only in a small number of studies showing either loss or gain of function effect for the mutants.[6,20,21]

Here, we describe the clinical findings of five CPHD patients with or without extra-pituitary manifestations, carrying *GLI2* mutations. A functional study was conducted on the missense mutations identified in our patients to assess their pathogenic role.

2 | SUBJECTS AND METHODS

2.1 | Patients

One hundred forty-five CPHD patients (88 male/57 female) were recruited from different Italian centres for diagnostic purposes. Of the 145 unrelated patients, seven had at least one affected relative (familial cases). They presented with a clinical and hormonal evidence of childhood-onset GH deficiency combined with at least one other pituitary defect in the absence of an identified cause of hypopituitarism (eg, cerebral tumours, cranial trauma, documented asphyxia, or other injuries at delivery). All the patients had been previously screened for mutations in the coding sequences of genes associated with multiple pituitary hormone dysfunctions (*PIT1*, *PROP1*, *HESX1*, *LHX3* and *LHX4*) and tested negative (data not shown).

The mean height of these patients at diagnosis was −2.58 ± 1.61 SDS and the mean delay in bone age relative to chronological age was 2.48 ± 2.05 years. GHD was present in all the patients, TSH deficiency in 70.3% (102/145) and ACTH deficiency in 50.3% (73/145) of the subjects. One hundred five subjects were prepubertal at the time of diagnosis. Among the remaining 40 subjects that could be evaluated in terms of pubertal age, 34 (85%) presented with FSH/LH deficiencies.

Morphological evaluation of the hypothalamus-pituitary area and/or of the central nervous system was performed by magnetic resonance imaging (MRI), using precontrast coronal spin-echo T1-weighted images followed by postgadolinium T1-weighted imaging. We obtained MRI data from 107 patients (74% of the total) and found abnormalities in 88 (82%) subjects; in particular anterior pituitary hypoplasia or aplasia was the most frequent abnormality and was present in 48 patients (45%), while pituitary stalk interruption and/or neuropituitary ectopia were observed in 31 patients (29%), 16 of them presenting both abnormalities.

Twelve patients presented also extra-pituitary abnormalities such as SOD, polydactyly, other midline defects or cerebellar abnormalities.

Patients or parents of the patients under 18 years of age gave their written-informed consent to participate to this study.

2.2 | Genetic analysis of the *GLI2* gene

Genomic DNA from patients and controls was extracted from peripheral blood leucocytes using standard procedures. The entire coding region of *GLI2* (13 exons and exon-intron boundaries) was amplified using previously described primers[17] with modifications, in 17 separate fragments (see Supporting information, Table 1). The PCR products were visualized on a 2% Agarose

TABLE 1 *GLI2* variations identified in the present study and the in silico analysis

Variants		dB SNP reference	Allele frequency	PolyPhen[a]	PROVEAN[b]	SIFT[c]
p.Pro386Leu	c.1157 C>T	rs757467621	0.00002 (3/119990)	Possibly damaging (0.923)	Deleterious (−3.929)	Tolerated (0.06)
p.Tyr575His	c.1723 T>C	rs763503195	Not available	Probably damaging (1.000)	Deleterious (−4.900)	Damaging (0.00)
p.Ala593Val	c.1778 C>T	rs977585784	Not available	Probably damaging (0.999)	Deleterious (−3.920)	Tolerated (0.21)
p.Val1111Glyfs*19	c.3332delT	Not reported	NA	NA	NA	NA
p.Arg1226*	c.3676 C>T	Not reported	NA	NA	NA	NA

NA, Not Applicable.
[a]PolyPhen score 1.0 is predicted to be "probably damaging".
[b]PROVEAN score less than −2.5 are predicted to be deleterious.
[c]SIFT score less than 0.05 are predicted to be deleterious, while those greater than 0.05 are neutral.

gel and purified using EXO-SAP PCR Enzymatic Clean-up kit (Euroclone, Milan, Italy). The purified products were then sequenced with Big Dye Terminator kit (Applied Biosystems, Foster City, CA, USA) and automatic sequencer ABI PRISM 3100 Genetic Analyzer (Applied Biosystems).The frequency of the identified variations in the general population was checked in the Exome Aggregation Consortium (ExAC browser, http://exac.broadinstitute.org) and 1000 Genomes project consortium database (http://www.1000genomes.org/).[22]

2.3 | In silico analysis

The impact of the variants were analysed by three different prediction tools: PolyPhen-2 (Polymorphism Phenotyping v2, http://genetics.bwh.harvard.edu/pph2/) SIFT (Sorting Intolerant From Tolerant, http://sift.jcvi.org) and PROVEAN (Protein Variation Effect Analyzer, http://provean.jcvi.org/index.php). The default settings were used in all the prediction tools.

2.4 | Functional analyses

2.4.1 | Plasmids

The expression vector pCS2 containing cDNA carrying either the full-length *GLI2* (*GLI2*FL-WT) or *GLI2* lacking the N-terminal repressor domain (*GLI2*ΔN-WT) were used to evaluate the transcriptional activity of the two different GLI2 isoforms on the expression of the luciferase reporter gene. The plasmids *GLI2*FL-WT and *GLI2*ΔN-WT were a gift from Erich Roessler (Addgene plasmid #17648 and #17649).

Variants were introduced into the wild-type cDNA using QuickChange XL site-directed mutagenesis kit following the manufacturer's instructions. The primers used for the mutagenesis are shown in Table S2. Reaction mixtures were denatured at 94°C for

5 minutes, cycled 25 times at 94°C for 15 seconds, 58°C for 1 minutes, 68°C for 8 minutes. After the reaction, the products were digested with DpnI. DH5a competent cells were transformed with the different constructs and grown on Luria Broth/ampicillin media. After selecting the correct clones by colony PCR, the plasmid DNA was isolated using Maxiprep kit (Qiagen, Milan, Italy). The mutant cDNAs were sequenced to confirm that the mutations were introduced as predicted.

A pGL3 vector containing a eightfold repeat of the 8x3-GliBS–LucII from Hnf3 floor plate enhancer in front of the chicken-crystallin minimal promoter that drives expression of the firefly luciferase (8x3-GliBS–LucII)[23] was obtained from RIKEN BioResource Center and used as the reporter plasmid in cotransfection. pRL-tk vector expressing the *Renilla* luciferase was used to normalize transfection efficiency and cell number.

2.4.2 | Cell culture and Luciferase assay

The NIH-3T3 (Mouse Embryonic fibroblast) cell line was cultured under standard conditions: DMEM High Glucose (Gibco-ThermoFisher Scientific, Monza, Italy) supplemented with 10% foetal calf serum and 1% Penicillin/Streptomycin. Transfections were carried out using Lipofectamine 2000 transfection reagent (Gibco-ThermoFisher Scientific) and were performed in 24-well plates.

NIH3T3 cells were seeded onto 24-well plates at 4×10^4 cells/well, 24 hours prior to transfection. The cells were cotransfected with the expression vector containing either wild type or mutant *GLI2*-cDNA and the reporter plasmid 8x3-GliBS–LucII. For testing, the transcriptional activity driven by the individual GLI2 plasmids (either wild type or mutants) 250 ng of plasmid DNA were used. When we used both the wild type and one of the mutants to evaluate the heterozygous condition, 125 ng of each plasmid was added. A control plasmid (pRL-TK, 50 ng) was included in each reaction to normalize for transfection efficiency.

Two days after transfection, growth media were removed and cells were washed gently with phosphate buffered saline. Passive lysis buffer (Promega, Madison, WI, USA) 100 μL/well was added and with gentle rocking for 15 minutes at room temperature cell lysates were harvested for DLR assay. The activities of firefly and Renilla luciferase were measured using the dual-luciferase reporter assay system (Dual-Glo Luciferase Assay System, Promega) according to the manual of the manufacturer. For each luminescence reading, after injector dispensing assay reagents into each well, there would be a 2-second premeasurement delay, followed by a 10-second measurement period. About 10 μL of cell lysate were transferred in white opaque 96-well plate. The luminescence obtained for the mutated and wild type constructs were normalized with the internal control Renilla luciferase signal and the activity of the mutated constructs was reported as percentage with respect to the wild type. Each experiment was performed in triplicate, and three independent experiments were performed. Quantitative data of the reporter gene assay are calculated as mean ± SEM. Student's *t* test was used to determine significant differences in each mutated construct compared to the wild type construct.

3 | RESULTS

3.1 | Molecular analysis of GLI2

Sequencing of exons and exon/intron boundaries of *GLI2* led to the identification of five variations in five unrelated CPHD patients. The mutations were three missense (p.Pro386Leu, p.Tyr575His, p.Ala593Val), one frameshift (p.Val1111Glyfs*19) and one nonsense (p.Arg1226X; Table 1). Of the missense variants, p.Tyr575His and p.Ala593Val were located in the Zinc-finger domain whereas p.Pro386Leu was located in the N-terminal repressor domain (Figure 1).

The potential functional impact of these missense variants was evaluated by three publicly available in silico tools: PolyPhen-2, SIFT and PROVEAN. All the variants were predicted to be deleterious by both PolyPhen and PROVEAN, whereas p.Tyr575His was predicted deleterious by SIFT (Table 1). These variants were either absent or present with an extremely low allele frequency (0.0001735, p.Pro-386Leu) in the Exome Aggregation Consortium (ExAC browser, http://exac.broadinstitute.org). The mutations p.Val1111Glyfs*19 and p.Arg1226X that create a premature stop codon is predicted to generate proteins lacking considerable portion of the C-terminal activation domain.

3.2 | Clinical characteristics of the patients carrying GLI2 variants

3.2.1 | Patient A (p.Pro386Leu)

This patient was a 4.9 years old girl born at term with appropriate length for gestational age. The parents were nonconsanguineous with normal stature. The height at the diagnosis was −2.6 SDS. After biochemical evaluations which excluded organic diseases, two consecutive stimulation tests for GH secretion were performed with Arginine (Arg) and Insulin Toleration Test (ITT) showing severe GH deficiency (peaks: 0.2 ng/mL and 0.5 ng/mL, respectively). The patient also showed deficiency of basal TSH (7.1 μU/mL). The MRI of the pituitary region performed with narrow scanning and gadolinium injection, reported a normal anatomy of the region with the sagittal T1 images showing only a mild hypoplasia of the pituitary gland. No extra-pituitary features were present. The analysis of the parents DNA revealed that this mutation was inherited from her mother with normal stature.

3.2.2 | Patient B (p.Tyr575His)

This patient was a girl affected by multiple pituitary hormone deficiency (GH, TSH and ACTH) diagnosed at 25 months of age. She was born at term from nonconsanguineous parents with no perinatal
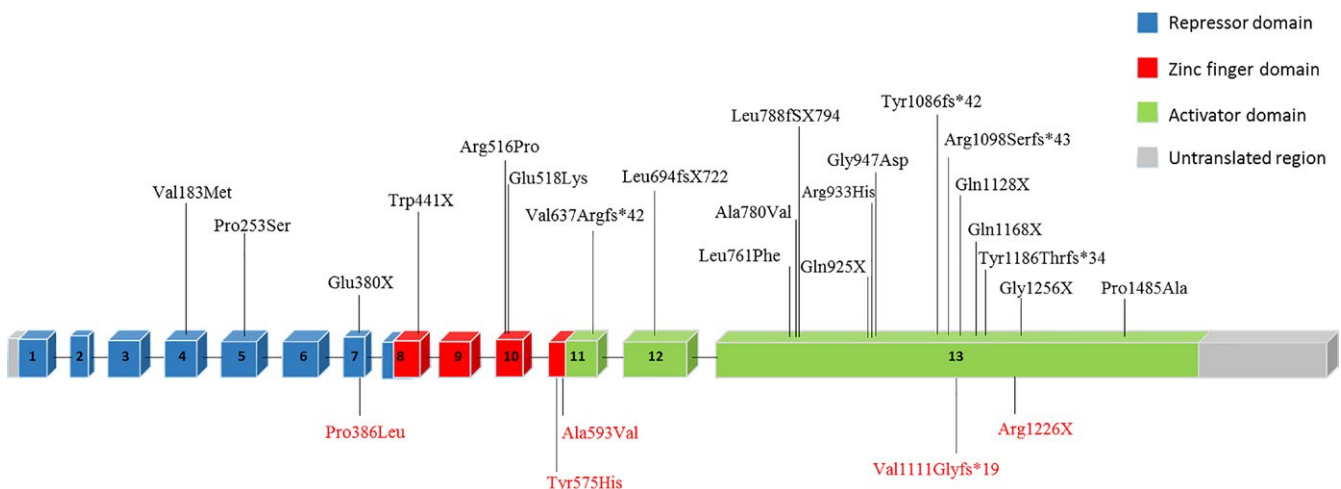
FIGURE 1 Schematic representation of *GLI2*. Mutations reported in the previous reports as likely pathogenic are indicated in black above the GLI2 protein diagram. Mutations identified in this study are indicated below the *GLI2* gene

complications and a birth weight of 2900 g. The bone age was delayed by 1.8 years at diagnosis and the height was −2.2 SDS. ACTH deficiency was diagnosed by an inadequate increase of ACTH (21 pg/mL) and cortisol (75 ng/mL) concentrations during hypoglycaemia. TSH was low normal 1.6 mU/L associated with low T4 (5.5 ng/mL) and FT4 (8.0 pg/mL). GH deficiency was diagnosed by peak GH of 0.2 and 0.1 ng/mL after L-DOPA and Arginine test, respectively.

Patient's pituitary and cerebral imaging showed anterior pituitary hypoplasia. Extra-pituitary manifestations were polydactyly, craniofacial abnormalities, bilateral renal hypoplasia and hypercholesterolaemia. The mother and a maternal uncle were also affected by familial hypercholesterolaemia and in the child hypercholesterolaemia persisted even after GH, levothyroxine and hydrocortisone replacement therapy.

The father was of normal stature, whereas the mother presented short stature (−2.4 SDS) and polydactyly. As expected, the mutation was inherited from the affected mother.

3.2.3 | Patient C (p.Ala593Val)

This patient was a male of 3.5 years old treated with recombinant GH therapy. He was born at term and appropriate for gestational age from normal stature nonconsanguineous parents. The height at the time of the diagnosis was −2.1 SDS. Growth hormone deficiency was revealed by double stimulation test with Arg and GHRH (peak: 2.4 ng/mL). ACTH deficiency was diagnosed for the low ACTH and cortisol level (31 pg/mL and 16.4 ng/mL, respectively) concentrations observed during hypoglycaemia. TSH was low (2.7 μU/mL) with low T4 (0.8 ng/ml) and FT4 (1.9 pg/ml). The patient did not show extra-pituitary features and MRI did not reveal abnormalities. Unfortunately, the DNA of the parents was not available for the genetic analysis.

3.2.4 | Patient D (p.Arg1226X)

This patient was a 3 years old male, born at term with appropriate for gestational age, the healthy parents were nonconsanguineous with normal stature. The height at the diagnosis was −1.8 SDS. The patient was diagnosed as CPHD following the result of GH double stimulation test with Arg/GHRH (peak: 3.9 ng/mL) and of the other hormone dosages (ACTH basal levels, 8.2 pg/mL; cortisol, 11 ng/mL; PRL, 4.54 ng/mL; LH 0.6 mU/mL, FSH <1 mU/mL). The pituitary and cerebral imaging showed anterior pituitary hypoplasia with no extra-pituitary features. The mutation was absent in the parents and thus arose "de novo" in this patient.

3.2.5 | Patient E (p.Val1111Gfs*19)

This case was a 16.6-year-old girl, born from nonconsanguineous healthy parents. She presented a polymalfomative syndrome characterized by complex congenital heart disease (pulmonary stenosis, DIA, mitral and tricuspid valvulopathy), renal hypoplasia with bladder -ureteral reflux, labiopalatoschisis, mental retardation, deafness and visual impairment (myopia with astigmatism). For these reasons, she underwent several surgical corrective procedures.

During the first days of life, she presented repeated hypoglycaemic crises. The GH peak of 0.5 ng/mL after stimulation test with insulin, the low serum cortisol (40 ng/mL) and free thyroxine levels (6.0 pmol/L) with normal TSH (2.8 UI/mL), allowed at the age of 40 days the diagnosis of CPHD. A replacement therapy with L-thyroxine, hydrocortisone and GH was initiated for a lifetime. The neuroradiological picture highlighted the stalk interruption syndrome with ectopy of the neurohypophysis and hypoplasia of the anterior pituitary.

At the age of 12 years, she started puberty induction therapy with the appearance of the menstrual cycle at the age of 13.6 years. She reached the final height compatible with the target height. The neuropsychiatric evaluation revealed intellectual disability (IQ = 52).

At the age of 16.5 years, after discontinuation of GH therapy for 3 months, GH secretion was re-evaluated (ITT-Insulin Tolerance Test) which confirmed the persistence of GH deficiency (GH peak <0.5 ng/mL; IGF-1 43 ng/mL) and the need for hormone lifelong replacement therapy.

The analysis of the parent's DNA revealed that the identified mutation was "de novo".

3.3 | In vitro analysis of the non-synonymous mutations

For the nonsense and frameshift mutations (p.Arg1226X and p.Val1111Glyfs*19) no functional assay was performed as they are expected to produce C-terminal truncated non-functional protein. In a previous report, similar mutations leading to truncated protein (p.Gln1256X and p.Tyr1086fs*42) exhibited strong dominant negative activity.[6] As the truncating mutations detected in our patients (Figure 1) are located in the proximity of those already tested, it is likely that they have a similar impact.

To evaluate if the here detected missense variations had an effect on the basic properties of GLI2, luciferase reporter assays were performed. Two series of plasmids, one bearing either wild type or mutant GLI2-cDNA and the other bearing the luciferase reporter gene were used. The plasmids bearing the GLI2-cDNA were of two types: one with the full-length GLI2 (GLI2FL-WT) and the other with GLI2 missing the N-terminal repressor domain (GLI2ΔN-WT), both inserted in the pCS2-MT vector. The optimized reporter plasmid 8x3-GliBS–LucII provides the GLI-binding site containing promoter that drives the expression of the firefly luciferase. The binding of the GLI2 to the binding sites drives the Luc II gene transcription and the luminescence correlated with functional activity of the assayed GLI2 protein.

In a preliminary experiment, the GLI2ΔN-WT induced up to 10-fold reporter activity (P < 0.0001) compared with GLI2FL-WT (Figure S1) due to the absence of the repressor domain, in line with the previous reports.[6,20]

The first set of experiments was performed by cotransfecting one type of GLI2-cDNA plasmid, either mutagenized or wild type,
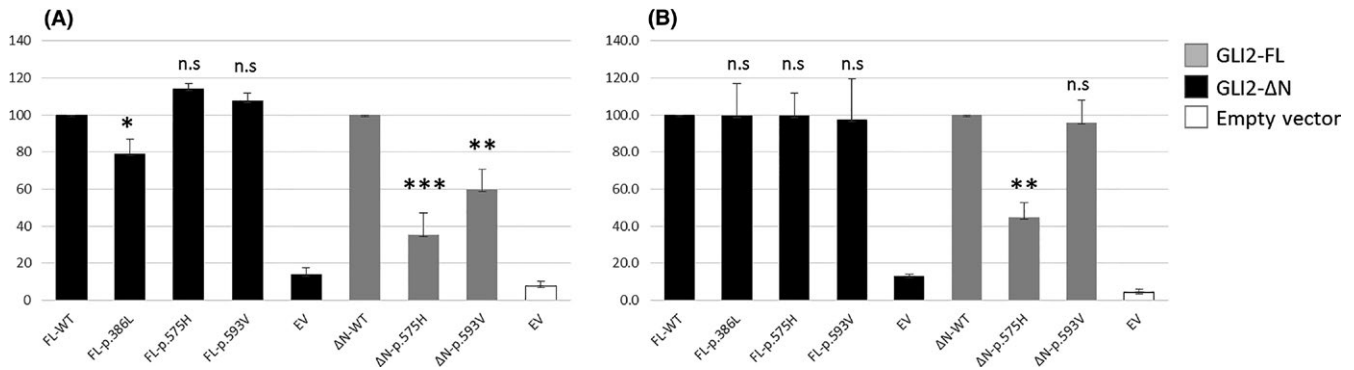
FIGURE 2 Expression vectors for mutated GLI2 and wild types proteins were transiently cotransfected into NIH-3T3 cells with a luciferase reporter gene under the control of an 8-fold repeat of the Hnf3β GLI-binding site. A, Activity of the variants individually. B, Activity of each variant when cotransfected with wild-type construct. ns - $P > 0.05$, $*P ≤ 0.05$, $**P ≤ 0.01$, $***P ≤ 0.001$

with the reporter plasmids (Figure 2A). The transcriptional activity of the plasmids carrying the full-length cDNA with the three missense mutations was assayed. Plasmids carrying p.575His and p.593Val did not show a different transcriptional activity in the presence of the repressor domain. On the contrary, the plasmid carrying p.386Leu showed a slightly reduced but significant difference in driving the luciferase activity in comparison to the wild type (Figure 2A).

When the transcriptional activity was tested for the plasmids containing the *GLI2*-cDNA lacking the repressor domain it was significantly reduced for the two mutants *GLI2*FL-p.575H and *GLI2*FL-p.593V of about 65% ($P < 0.0001$) and 40% ($P < 0.0003$) in comparison to the wild type, respectively (Figure 2A). Obviously, for the p.Pro386Leu mutation, located in the repressor domain the assay was not performed for the *GLI2*ΔN construct that lacks this domain.

In a second set of experiments, all the *GLI2*-cDNA full length and ΔN constructs were cotransfected with an equal amount of wild-type *GLI2* plasmid to reproduce in vitro the heterozygous condition of the patients that carry the 50% of the mutated alleles. The *GLI2*ΔN bearing the p.575H mutation still maintained a reduced activity of about 55% ($P = 0.0003$) when cotransfected with the wild type (Figure 2B).

4 | DISCUSSION

The exact frequency of *GLI2* mutations in patients with hypopituitarism is difficult to establish due to the large phenotypic variability of the studied cohorts.[17-19,21,24] Moreover, *GLI2* is a large and highly polymorphic gene with several rare variations reported in public databases making the establishment of the variants pathogenicity difficult without performing functional studies. To date, in vitro testing of *GLI2* variants has been performed in a very limited number of studies.[20,21] Roessler et al[6] through a luciferase-based functional test demonstrated undetectable transcriptional activity and a strong dominant negative effect for the mutants p.Tyr1086fs*42 and p.Gln1256X which produce proteins with truncated carboxyl domain.

In the present study, we report five unrelated subjects bearing five non-synonymous *GLI2* variations in a cohort of 145 CPHD patients

with or without extra-pituitary findings all at the heterozygous state. All the variants were absent in the public databases or if present, the frequency was not reported or extremely low (0.0001735) and predicted to be pathogenic by at least one prediction software (Table 1). The frameshift and nonsense mutations (p.Val1111Gfs*19 and p.Arg1226X, respectively) were both located within the C-terminal transactivation domain similarly to the truncating mutations p.Tyr1086fs*42 and p.Gln1256X that exhibited a dominant negative effect in vitro in the previous study.[6] Thus it is conceivable that the variants identified in the present study exert the same effect.

On the contrary in the absence of a functional test, the pathogenic significance of the here detected missense variations remains unknown and we can only speculate about their role solely on the basis of their rarity and in silico prediction. In order to shed light on the potential damaging effect of these three missense mutations, we performed an in vitro luciferase assay.

Introduction of p.Pro386Leu mutation located within the GLI2 repressor domain reduced the luciferase activity of about 20% compared to the wild type construct (Figure 2). The reduced level of transcriptional activity might be due to an enhanced repressor activity conferred by this variant.

The most striking results were observed for the *GLI2*ΔN-p.575H and *GLI2*ΔN-p.593V plasmids that revealed a significant reduction compared with the *GLI2*ΔN-WT ($P < 0.0001$ and $P < 0.0003$ respectively; Figure 2). Thus in the absence of the repressor domain, these two mutations exerted a high inhibitory effect solely in the context of activation as GLI2 acts as transcriptional activator or repressor depending on the isoform. Both the variations reside within the fifth zinc finger of the GLI2 DNA-binding domain, which consists of five consecutive Cys_2His_2 fingers of which motifs 4 and 5 make the strongest base contacts with the DNA. Moreover, it has also been reported that p.575Tyr is critical in making hydrogen bond with the DNA.[25] Thus, these variants could strongly affect the binding to the DNA and consequently reduce the transcriptional activity. The mutagenized constructs were then cotransfected with an equal amount of wild-type plasmids to reproduce the heterozygous condition of the patients that carry the 50% of the mutated alleles. A significant reduction in the luciferase transcriptional activity was maintained

for the mutant p.575His (Figure 2) indicating a possible dominant negative effect of this mutant construct on the wild type.

By combining the data from the literature,[6,20,21] the in silico prediction (Table 1) and the functional results a probably damaging effect can be assessed for at least four of the five here described variants. The pathogenic significance of p.Pro386Leu is still difficult to determine from our results. This variant is also reported in the ExAc Consortium database with an extremely low frequency (0.0001735, reported three times in the latino population) although the presence of low-frequency variants in the public databases such as ExAC does not exclude a pathogenic role as they include also patients. Moreover, the variation was inherited from the patient's mother with normal stature. Thus, p.Pro386Leu can be classified as a variant with unknown pathogenic significance and incomplete penetrance, that is typical for GLI2 mutation.[19,26]

The exact frequency of GLI2 pathogenic mutations in CPHD patients without extra-pituitary features is still difficult to determine because of the phenotypic heterogeneity of the studied cohorts and the lack of proof of pathogenicity for many GLI2 variants reported in those cohorts. Among the 281 patients screened in previous studies for GLI2 in considerably large cohorts presenting only congenital hypopituitarism without other manifestations such as holoprosencephaly or midline facial defects,[18,20,21] 30 patients (7.8%) have been reported carrying non-synonymous variations. However, if we consider the variants with a frequency less than 0.001 in the public databases and those with pathogenic evidence proved by functional studies, only seven variants (p.Glu518Lys, p.Pro253Ser, p.Ala-780Val, p.Arg933His, p.Gly947Asp, p.Pro1485Ala and p.Arg516Pro; Figure 1) corresponding to a global frequency of 1.8% can be considered "likely pathogenic." In the present study, among the 133 index cases presenting CPHD without severe extra-pituitary features, three patients (2.3%) carried mutations (p.Pro386Leu, p.Ala593Val and p.Arg1226X) similarly to the other cohorts.

In conclusion, the analysis of GLI2 in individuals with CPHD led to the identification of five variations which were not previously described in patients with hypopituitarism. Here, we provided pathogenic evidence for variations p.Tyr575His and p.Ala593Val based on the functional evaluation.

This study confirms that GLI2 is an important candidate for CPHD also in absence of extra-pituitary phenotypes. As the GLI2 gene is highly polymorphic with many missense mutations of uncertain significance, functional assays are important for the evaluation of their pathogenic role.

CONFLICT OF INTEREST

Nothing to declare.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.