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Clinical Research Article

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Comprehensive Identification of Pathogenic Gene Variants in Patients With Neuroendocrine Disorders

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Abstract

Purpose: Congenital hypopituitarism (CH) can present in isolation or with other birth defects. Mutations in multiple genes can cause CH, and the use of a genetic screening panel could establish the prevalence of mutations in known and candidate genes for this disorder. It could also increase the proportion of patients that receive a genetic diagnosis.
Methods: We conducted target panel genetic screening using single-molecule molecular inversion probes sequencing to assess the frequency of mutations in known hypopituitarism genes and new candidates in Argentina. We captured genomic deoxyribonucleic acid from 170 pediatric patients with CH, either alone or with other

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abnormalities. We performed promoter activation assays to test the functional effects of patient variants in *LHX3* and *LHX4*.

Results: We found variants classified as pathogenic, likely pathogenic, or with uncertain significance in 15.3% of cases. These variants were identified in known CH causative genes (*LHX3, LHX4, GLI2, OTX2, HESX1*), in less frequently reported genes (*FOXA2, BMP4, FGFR1, PROKR2, PNPLA6*) and in new candidate genes (*BMP2, HMGA2, HNF1A, NKX2-1*).

Conclusion: In this work, we report the prevalence of mutations in known CH genes in Argentina and provide evidence for new candidate genes. We show that CH is a genetically heterogeneous disease with high phenotypic variation and incomplete penetrance, and our results support the need for further gene discovery for CH. Identifying population-specific pathogenic variants will improve the capacity of genetic data to predict eventual clinical outcomes.

Key Words: congenital hypopituitarism, genetic screening, variants, single molecule molecular inversion probes

Birth defects that interfere with craniofacial development can result in significant morbidity and can include cognitive, neurosensory, and neuroendocrine defects that create life-long burdens for care. Genetic defects that disrupt the development of the forebrain, midbrain, hindbrain, 5 facial prominences, and pituitary gland can cause a spectrum of disorders that range from holoprosencephaly (HPE) and septo-optic dysplasia (SOD) to pituitary hormone deficiencies with no other obvious abnormalities (1).

Hypopituitarism is a disorder characterized by a shortage of growth hormone (GH) and at least 1 other pituitary hormone. When GH is the only deficient hormone, a diagnosis of isolated GH deficiency (IGHD) is made, whereas if 2 or more pituitary hormones are involved it is called combined pituitary hormone deficiency (CPHD). About 45% of IGHD cases evolve into CPHD after 5.4 years (2, 3). These disorders are estimated to have a prevalence of 1 in 4000 individuals worldwide (4). CPHD is caused by both genetic and nongenetic factors (5).

Genetic causes typically manifest in early childhood with insufficient hormone secretion and abnormal anterior pituitary gland development. Sporadic cases with no family history are the most common, and familial cases (~10%) are more likely to have a genetic diagnosis (6), although other genetic background and environmental factors likely influence the penetrance or expressivity of the features. Congenital hypopituitarism (CH) is considered syndromic if other craniofacial structures and neurosensory systems are affected and nonsyndromic if only the pituitary gland and its target organs are compromised. Diagnosis is challenging, particularly in the neonatal period.

The genetic basis for CH and related disorders is emerging, with over 30 genes known to cause CH (4). *PROP1* was reported to be the most common known cause of CPHD, accounting for an average of 11% of total cases worldwide (6–8). Very few systematic screens testing multiple genes have been carried out. Instead, studies in large cohorts of CPHD patients were focused on screening variants in single genes. Retrospective analyses of the literature suggest that 16% and 11% of the cases of CPHD and IGHD respectively, can be explained by mutations in the most frequently screened 5–6 genes: *HESX1* (OMIM reference *601802), *PROP1* (*601538), *POU1F1* (*173110), *LHX3* (600577) and *LHX4* (602146) (6, 9–12). However, these estimates may represent an ascertainment bias, as negative screening results may not be published. In addition, the rate of genetic diagnosis is skewed in some population groups by 2 common, founder effect mutations in *PROP1* that originated in Eastern Europe and Spain or Portugal (8). For example, Budny et al screened for the same 5 genes in 80 Polish patients and solved 40% of the cases, but all of the mutations were either the *PROP1* 301_302delAG or 150delA (13). Unbiased population-level screening data on the genetic basis of disease are lacking.

It is noteworthy that the causal genes for HPE, SOD, and CH overlap. For example, the phenotypic features of SOD or CH can be caused by mutations in *HESX1*, *OTX2* (*600037), *SOX2* (*184429), *SOX3* (*313430), and *PAX6* (*607108). Mutations in genes implicated in Kallmann syndrome (KS), like *FGFR1* (*136350), *PROKR2* (*607123), and *FGF8* (*600483) have been reported in patients with SOD (14, 15), and *FGF8* was also implicated in an HPE case (16). Disruption of the sonic hedgehog (SHH) signaling pathway can cause HPE and/or CPHD (17–19). The most common genes implicated in IGHD are those encoding GH (*GH1*) (*139250) and the GH-releasing hormone receptor (*GHRHR*) (*139191). Also, IGHD is sometimes caused by mutations in genes involved in early

embryonic development, like *OTX2, HESX1, SOX2,* and *SOX3* (1, 20).

In this study, we utilized a cost-effective approach that allows the capture of coding exons of 67 candidate genes for CPHD, IGHD, HPE, and SOD from several individuals in a single reaction (21). We captured 693 coding exons of 30 known genes and 37 new candidate genes from genomic deoxyribonucleic acid (DNA) using a custom designed panel of small molecule molecular inversion probes followed by sequencing (smMIPS). This smMIPS technique is a targeted sequencing method amenable to screening panels of genes in large cohorts for germline or somatic variants. It uses pools of oligonucleotide probes with sequences complementary to the targeted genes' exons. After hybridizing to the targeted region, these probe ends face inward from 3' to 5', resulting in a gap that is filled by DNA polymerase and sealed by a ligase. A universal primer pair is used to amplify all of the resulting captured sequences though homology to the smMIPS backbone, generating a sequencer-ready library without the need for separate library preparation steps such as DNA fragmentation and adaptor ligation. This step also adds sample-specific index sequences, allowing for hundreds of samples to be combined for sequencing in a single batch. This technology is cost-efficient, high-throughput, sensitive, and specific, and can scale to thousands of target regions across hundreds to thousands of patients (22–24).

We aimed to find genetic causes of CH in a cohort of 170 pediatric patients diagnosed with CPHD or IGHD, with or without other abnormalities (64% and 36% of the cases, respectively). We identified novel pathogenic (P), likely pathogenic (LP), or variants with uncertain significance (VUS) in 26 cases. These variants were found in known causative genes and in new candidate genes. This study establishes an inexpensive tool for screening in areas where exome sequencing may be cost prohibitive, and it establishes for the first time, the incidence of pathogenic variants in Argentinean patients with CH.

Methods

Patient recruitment

Informed parental consent, patient assent and approval by the Ethics Committee from the Hospital de Niños Ricardo Gutiérrez and Hospital Garrahan, Buenos Aires, Argentina, were obtained. The University of Michigan Institutional Review Board approved the analysis of anonymized DNA samples.

Patients were diagnosed with hormone deficiencies according to previously described and internationally accepted criteria by the Endocrine divisions of 2 major pediatric hospitals in Buenos Aires (21). Patients were diagnosed with GH deficiency (GHD) on the basis of abnormally low growth velocity and GH peak lower than 4.8 µg/L after sequential arginine/clonidine pharmacological stimulation tests. Thyroid-stimulating hormone (TSH) deficiency was diagnosed in individuals with free thyroxine <1.0 ng/dl with low or normal TSH levels, TSH≤10 mU/L in patients under 2 months of age and ≤6.5 mU/L in older infants. Adrenocorticotropic hormone deficiency was diagnosed based on low basal serum cortisol, <30.3 nmol/L in patients under 2 months of age, <58 nmol/L in patients between 2 and 6 months, and <165 nmol/L in older infants (22). Prolactin (PRL) deficiency was considered in individuals with serum levels <2.5th centile for sex and age. Central diabetes insipidus was diagnosed when polyuria was associated with a urinary:plasma osmolarity ratio of <1.5 and the patient had a plasma osmolality>300 mosm/L. Gonadotropin deficiency was diagnosed in boys aged between 15 days and 6 months when serum luteinizing hormone (LH) and testosterone were <5th centile, <0.8 IU/L and <30 ng/dl, respectively. In girls from the age of 15 days to 2 years, gonadotropin deficiency was assumed when follicle-stimulating hormone (FSH) levels were <1.0 IU/L (22, 23). In older patients, gonadotropin deficiency was defined as delayed or absent pubertal development with a low serum testosterone (< 3.47 nmol/L) associated with inappropriately low or normal LH and FSH levels. CPHD was defined as the presence of hormone deficiency affecting at least 2 anterior pituitary hormone-producing cell types. Magnetic resonance imaging (MRI) of the brain and the hypothalamic-pituitary area was performed in all patients.

Whole blood was collected from 170 patients diagnosed with IGHD or CPHD belonging to 168 unrelated families. Part of this cohort (58 cases) was analyzed previously as a test run for assessing the quality of the panel, and we reported cases with a *GH1* mutation (21). The complete results are included for the first time in this report.

Genomic DNA isolation

Genomic DNA was extracted from peripheral blood cells, as previously described (21). Briefly, Puregene Blood kit (Qiagen, Hilden, Germany) was used according to the protocol provided by the manufacturer. The DNA was quantified using QuantiFluor dsDNA System (Promega, Madison, Wisconsin, United States) and DNA concentration was normalized to 25 ng/µl for smMIPS assay. The absorbance ratio 260/280 nm was used to assess the purity of DNA. A ratio between 1.8 and 2.1 was used as an inclusion criterion for DNA sample processing in following steps.

Molecular studies

A custom panel based on smMIPs was designed and synthesized to capture the coding exons of 67 known and putative hypopituitarism genes, from patients and available relatives' DNA, as previously described (21). Sequencing was performed on an Illumina HiSeq, San Diego, California, United States, and mapping to the human genome GRCh37 was performed by an in-house pipeline *mimips (*available at https://github.com/kitzmanlab/mimips). GATK haplotypecaller (https://gatk.broadinstitute.org/hc/en-us) was used to detect individual variants.

Variant prioritization

Resultant variant file (VCF) was annotated with databases dbSNP, gnomAD, and ClinVar, and several pathogenicity predictors such as Polyphen2 (http://genetics.bwh.harvard.edu/pph2/), SIFT (http://sift.bii.a-star.edu.sg/), Mutation Taster (http://www.mutationtaster.org/), CADD (https://cadd.gs.washington.edu/) and REVEL (https://sites.google.com/site/revelgenomics/about) were used to predict variant implications in function protein. Variant filtering and prioritization was performed on B_platform (https://www.bitgenia.com/b-platform/). Candidate variants were selected based on already reported pathogenic variants, moderate or high impact variants (missense, stop gained, indels), low or none allele frequency in control frequency databases and, predicted damaging effect. For healthy controls, we use the gnomAD database and Dr Martí's database of over 100 Argentinean controls (nonendocrine patients) derived from our recent project (http://apps.bitgenia.com/100exomas). Finally, we assessed variant pathogenicity using the American College of Medical Genetics (ACMG) guidelines for variant interpretation (24).

Plasmid constructs

Wild type (WT) human *LHX3a* and human *LHX4* cDNAs were cloned into the expression vector pcDNA3.1/myc-His6 (Invitrogen). To generate the mutated forms of hLHX3a (Leu220Met and Pro187Ser) and LHX4 (Gln100His, Trp204Leu and Arg84His), the QuickChange II site-directed mutagenesis kit (Stratagene; Agilent, Santa Clara, CA) was used following the manufacturer's instructions. The mutant complementary DNAs (cDNAs) were Sanger sequenced to confirm that the mutations were introduced. Reporter vectors encompassing sequences of the 5' proximal promoters of the human GH (-2446 to +20; +1 designates the transcription start site) and the human αGSU (-548 to -1) genes were obtained by Polymerase Chain Reaction using control human DNA as a template and primers containing the KpnI and HindIII restriction enzyme sequences required to clone them into the pGL3-luc plasmid (Promega): αGSU Fw: 5'-GGGGTACCCCAGAGCTGTGCAGGTTTTAGGG-3', αGSU Rv: 5'-CCCAAGCTTGGGGGGCTTTTTGCAGGATGTGT-3', GH1 Fw: 5'-GGGGTACCCCGGTCAGTGTTGGAACTGCAT-3', GH1 Rv: 5'-CCCAAGCTTGGGAGGACCCTGAGTGGTTCGG -3'. The PCR products were cloned into the pGL3-luc plasmid, transformed into DH5α *E. coli* and checked by Sanger sequencing.

Cell culture and transfection

HEK293T cells (ATCC CRL-11268) were grown in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum, 1% L-glutamine, and 1% penicillin/streptomycin (pH 7.4) at 37°C under 5% CO2 atmosphere. HEK293T cells were transfected using Lipofectamine 3000 reagent (ThermoFisher Scientific, Waltham, Massachusetts, United States) according to the manufacturer's instructions.

Luciferase assays

HEK293T cells were seeded onto 96-well plates at 1.5 x 10^4 cells/well. 24 hours later, cells were co-transfected with 30 ng of the human αGSU (LHX3 target) or hGH (LHX4 target) luciferase reporters, 15 ng of Renilla luciferase expression vector (pRL-SV40, Promega), and either 15 ng of pcDNA 3.1 empty vector (EV), WT, or mutant LHX3a/LHX4 expression vectors per well. To mimic the heterozygous condition, 7.5 ng of each plasmid (EV, WT, or variants) were used. Forty-eight hours after transfection, cell lysates were assayed for luciferase activity using the Dual-Luciferase Reporter Assay System (Promega) in a GloMax luminometer (Promega) according to the manufacturer's instructions. Firefly luciferase activity was normalized to Renilla luciferase activity. Results are shown as means ± standard deviation (SD) of 3 independent experiments performed in triplicate. Statistical analysis was performed using one-way ANOVA. $P < 0.05$ was considered statistically significant.

Western blot

Lysates were prepared from HEK293T cells subjected to transfections as described in each experiment. Cells were lysed in sterile Phosphate-buffered saline (PBS) with 1 mM phenylmethylsulfonyl fluoride protease inhibitors (Active Motifs, Carlsbad, California, United States), as indicated by the manufacturer. Lysates were centrifuged at 12000 g for 15 minutes at 4°C and the protein concentration was determined by Bradford protein assay.

Proteins were run on 10% sodium dodecyl sulphate–polyacrylamide gel electrophoresis and transferred onto a nitrocellulose membrane and were incubated with the following antibodies: Myc tag (#2272S; Cell Signaling Technology, Danvers, Massachusetts, United States) and GAPDH (6C5, sc-32233; Santa Cruz Biotechnology, Dallas, Texas, United States). An anti-rabbit-HRP (horseradish peroxidase) (#7074V) was used as a secondary antibody (Cell Signaling Technology). ECL (enhanced chemiluminescence) (#1705061; Clarity Western ECL Substrate) was used to detect proteins as indicated by the manufacturer's protocol. Images were obtained with G:Box (Syngene, India) and analyzed with ImageJ (NIH).

Immunofluorescence

HEK293T cells were grown in round glass coverslips, transfected with the indicated plasmids, and subsequently fixed and permeabilized with ice-cold methanol. Cells were washed and blocked in PBS 1% BSA for 30 minutes and then incubated overnight at 4°C with anti-Myc tag antibody (1/100). Coverslips were incubated with anti-rabbit Alexa 647 (#4414, Cell Signaling). Negative controls were carried out using PBS 1% BSA instead of the primary antibody. Nuclei were stained with DAPI 2 µg/ml (Sigma Aldrich, St. Louis, Missouri, United States). Photos were taken with an Olympus DSU IX83 Spinning Disk microscope.

Protein modeling

In silico protein structure analysis was performed when crystalized structures were available for the region of the protein harboring the potentially pathogenic variant. The structures for the LHX4 LIM and Homeobox domains (PDB 6CME and 5HOD, respectively) were downloaded from the PDB database. A model of the LHX3a homeobox domain was built from the crystal structure of the LHX4 homeobox using Modeller (Version 9.24 https://salilab.org/modeller/). Visualization and mutations were done using Maestro (Version 2019-4: Maestro, Schrödinger, LLC, New York, USA https://www.schrodinger.com/maestro). The amino acid change is visualized in the rotamer form that best maintains the orientation of the wild type residue in the crystal structure.

Protein conservation

Protein FASTA sequences from different species were retrieved from the National Center for Biotechnology Information repository and multiple alignment was performed using Clustal Omega (an online tool from The European Bioinformatics Institute [EMBL-EBI], http://www.ebi.ac.uk/Tools/ msa/clustalo/).

Results

Clinical features

To determine the prevalence of genetic variation contributing to CH, we collected 170 patients from non-consanguineous families in Argentina with a mean age of 5.7 ± 0.37 years at the age of diagnosis. A total of 113 had CPHD (66%) and 57 (34%) had IGHD (Table 1). Importantly, all cases were from Argentina, an understudied admixed population with contributions from Native American, European, and African ancestry (25–27). Nearly half of the patients (83/170) had morphological anomalies of the pituitary gland. All patients had a GH deficiency; the most frequent additional pituitary hormone deficiency was TSH (60%) followed by ACTH deficiency (41%). Associated phenotypes, including craniofacial

Table 1. Characteristics of the study subjects

Total patients	170
Age	
Median age (range)	5 (1–29)
Mean age	5,7
Gender	
Male	101 (60%)
Female	69 (40%)
Diagnosis	
IGHD	57 (34%)
CPHD	113 (66%)
Cases	
Familial	1 (3 patients)
Sporadic	167
Pituitary hormone deficiency	
GH deficiency	170 (100%)
ACTH deficiency	70 (41%)
TSH deficiency	102 (60%)
Gonadotropin deficiency	16 (9%)
PRL deficiency	8 (5%)
ADH deficiency	11 (6%)
MRI: pituitary stalk	
Absent	35
Thin	12
Interrupted	4
Normal	70
MRI: anterior pituitary	
Absent	5
Hypoplasia	83
Normal	50
MRI: posterior pituitary	
Absent	20
Ectopic	54
Hypoplasia	2
Normal	60

IGHD: Isolated Growth Hormone Deficiency; CPHD: Combined Pituitary Hormone Deficiency; GH: Growth Hormone; ACTH: Adrenocorticotropic hormone; TSH: Thyroid-Stimulating Hormone; PRL: Prolactin; ADH: Antidiuretic Hormone; MRI: Magnetic Resonance Imaging.

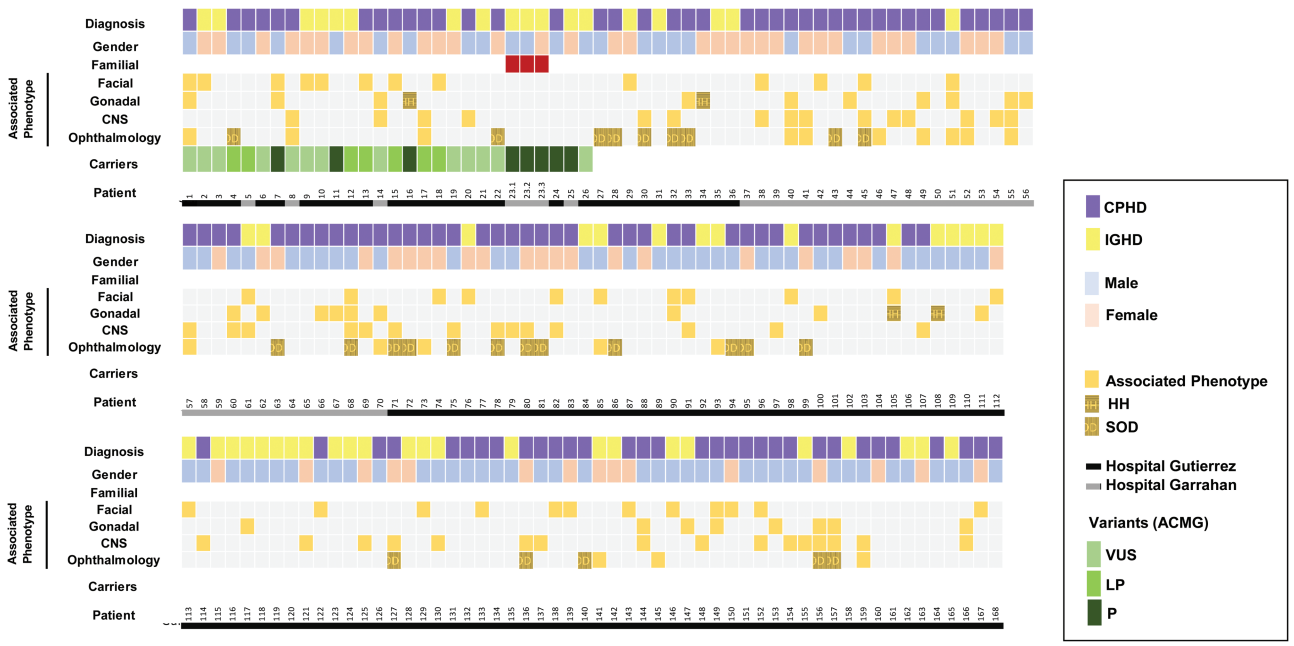

Figure 1. Patient information. A total of 170 patients with CPHD or IGHD were sequenced using an smMIP panel for 67 disease-associated genes. Samples are displayed in columns from left to right. Candidate variants were found in 26 cases (16%) from which 8% were unique mutations that met the American College of Medical Genetics (ACMG) criteria to be classified as P or LP and 8% were VUS. Abbreviations: ACMG, American College of Medical Genetics; CPHD, Combined Pituitary Hormone Deficiency; IGHD, Isolated Growth Hormone Deficiency; LP, Likely Pathogenic; P, Pathogenic; smMIP, small molecule Molecular Inversion Probes; VUS, Variant of unknown significance.

abnormalities, alterations of the gonadal axis, and/or optic pathway were observed in 64% of the studied patients (Fig. 1) (28).

smMIP sequencing panel

Genomic DNA from patients and relatives were captured using our custom smMIP panel, and next generation sequencing was conducted. Coding exons and intron-exon boundaries of 67 selected genes associated with CPHD, IGHD, SOD, and HPE in humans and/or mice were targeted (21). The mean sequencing depth was 700X per sample (29).

Evaluation of variants

Plausibly disease associated variants were found in 26 cases. Unique variants that met the ACMG criteria for pathogenicity or LP were identified in 13 cases (Fig. 1). Variants annotated in gnomAD with MAF > 0.05 or found in sequence data of in-house control group were excluded. Potentially pathogenic variants were classified in (1) genes most relevant to CPHD (Table 2); (2) less frequently reported genes in CPHD (Table 3) and (3) new candidate genes (Table 4). Interestingly, no cases of pathogenic *PROP1* mutations were found in this study even though average coverage was >100× for all 3 exons of this gene (29).

Variants in Well-established CPHD Genes

LHX3

Two novel, heterozygous variants of uncertain significance were identified in *LHX3* in 3 unrelated cases. *LHX3*:c.559C>T, p.Pro187Ser was found in patient 1 diagnosed with CPHD, and *LHX3*:c.658C>A, p.Leu220Met was found in patients 2 and 3, with IGHD alone or in combination with hypogonadism, respectively (Table 2, Fig. 2A–2B). These variants are at evolutionary conserved residues and are predicted to be damaging, suggesting that amino acid substitutions at these positions are detrimental to protein function (Fig. 2C), and they were absent from control databases.

Functional studies suggested that p.Pro187Ser is damaging, while p.Leu220Met may be tolerated. Protein modeling indicated that the *LHX3* p.Pro187Ser may disrupt the helix-turn-helix conformation of the homeodomain, probably destabilizing the DNA interaction domain (Fig. 2D). To determine the pathogenic effect of these variants, transcriptional activation assays were performed using the αGSU promoter coupled to a luciferase reporter gene. The *LHX3* p.Pro187Ser variant significantly impairs activation of the luciferase reporter gene, while the *LHX3* p.Leu220Met has WT activity on this reporter construct (Fig. 2E). Co-transfection of variant and WT expression vectors excluded dominant negative effects. Using immunofluorescence, we confirmed that

Table 2. Variants found in most relevant genes

Case	1	2	3	4	5	6	7	8	9	10	11
Age at time of diagnosis (years unless specified)	1 month	1	6	2	11	7	5	8	9	4	6
Gender	M	F	F	M	M	F	M	F	F	F	M
Hormone deficiencies	GH, TSH, ACTH, LH, FSH	GH	GH	GH, TSH, ACTH	GH, TSH, PRL, ACTH	GH, TSH	GH, TSH	GH, ADH (neonatal)	GH	GH	GH
Height SDS	-0.21	-3.51	-3.07	-0.1	-6.4	-2.06	-6.56	-2.4	-5.59	-5.1	-4.27
Pituitary imaging											
Anterior	Normal	Normal	Hypoplasia	Hypoplasia	Hypoplasia	Normal	No data	Hypoplasia	Hypoplasia	Hypoplasia	Normal
Stalk	Normal	Normal	No data	Absent	Normal	Normal	No data	Agenesia	Absent	Absent	Normal
Posterior	ectopic	Normal	No data	Ectopic	Normal	Normal	No data	Absent	Ectopic	Normal	Ectopic
Other	Optic nerve hypoplasia	–	–	Chiasm and papila hypoplasia	–	–	–	Aracnoid cyst, right cerebellar hemisfery hypoplasia.-	–	–	Empty sella turcica
Associated malformations and features	Micrognathia and depressed nasal bridge. Micropenis and bilateral cryptorchidism. Hypoglycaemia neonatal and jaundice.	Depressed nasal bridge, frontal bossing. SGA (weight SDS –2.72)	Hypogonadism	SOD	–	Ulnar valgus	Depressed nasal bridge, frontal bossing, myopia, micropenis, hypospadias	Myopia and astigmatism. Neonatal diabetes insipidus, speech neurodevelopmental delay (speech, fine motor), mild left foot paresia	Midfacial hypoplasia	Depressed nasal bridge, hypoplasia of nasal alae, short philtrum, microcephaly, and mild hearing loss	Thalassemia minor
Gene	LHX3	LHX3	LHX3	LHX4	LHX4	LHX4	GLI2	GLI2	OTX2	HESX1	HESX1
Position (GRCh37)	9:139090816G>A	9:139090 630G>T	9:139090 630G>T	1:180235 578G>T	1:1802409 74G>T	1:1802355 29G>A	2:121747 688G>GGCC ATGCCAT GGCT	2:121744 178C>T	14:57268 779C>G	3:57233 870A>G	3:572327 89G>A
Transcript	ENST00000 371746	ENST00000 371746	ENST00000 371746	ENST00000 263726	ENST00000 263726	ENST00000 263726	ENST00000 361492	ENST00000 361492	ENST00000 672264.2	ENST00000 473921	ENST00000 473921
Variant	c.559C>T	c.658C>A	c.658C>A	c.300G>T	c.611G>T	c.251G>A	c.4207_4220dup ATGG CTGCC ATGCC	c.2281C>T	c.568G>C	c.77T>C	c.349C>T
Protein	p.Pro187Ser	p.Leu220Met	p.Leu220Met	p.Gln100His	p.Trp204Leu	p.Arg84His	p.Ser1404fs	p.Leu761Phe	p.Ala190Pro	p.Ile26Thr	p.Gln117*

Table 2. Continued

Case	1	2	3	4	5	6	7	8	9	10	11
Type of Mutation	Missense	Missense	Missense	Missense	Missense	Missense	Frameshift	Missense	Missense	Missense	Stop gained
Genotype	Heterozygous	Heterozygous	Heterozygous	Heterozygous	Heterozygous	Heterozygous	Heterozygous	Heterozygous	Heterozygous	Heterozygous	Heterozygous
dbSNP	–	–	–	rs1156273617	–	rs374124070	–	rs1435272545	–	rs28936416	–
Polyphen	Probably damaging	Benign	Benign	Benign	Probably damaging	Probably damaging	–	Benign	Benign	Probably damaging	–
SIFT	Damaging	Tolerated	Tolerated	Damaging	Damaging, tolerated	Damaging	–	Damaging	Tolerated	Damaging	–
Mutation taster	Disease_causing	Disease_causing	Disease_causing	Disease_causing	Disease_causing	Disease_causing	–	Disease_causing	Disease_causing	Disease_causing	Disease_causing
CADD	28.7	20.7	20.7	24.5	25.3	25.6	26.9	31	24.5	24.6	37
REVEL	0.973	0.583	0.583	0.654	0.937	0.808	–	0.472	0.498	0.933	–
Allele frequency gnomad	Absent	Absent	Absent	0.00000398 (0 hom)	Absent	0.0000496 (0 hom)	Absent	0.000004013 (0 hom)	Absent	Absent	Absent
Score ACMG	VUS BP1, PM2, PP3	VUS BP1, PM2, PP3	VUS BP1, PM2, PP3	LP PM1, PM2, PP2, PP3, BP1	LP PM1,PM2, PP2,PP3	VUS PM1, PM2, PP2, PP3	P PVS1, PM2, PP3	VUS BP1, PM2, PP3	VUS PM2, PP2, PP3	VUS PM2, PP3, PP5	P PVS1, PM2, PP3 BP1
Family history	Not tested	Not tested	Not tested. Mother has short stature	Not tested	Absent in healthy father, mother not Tested	Present in healthy with mother, absent in the short stature, absent in Father	Present in mother Healthy father	Absent in healthy mother, father not Tested	Not tested	Present in healthy father, Absent in Healthy mother	Not tested

Human genome reference GRCh37/HG19 was used. gnomAD database v2.1.1 was used (https://gnomad.broadinstitute.org/).
Abbreviations: ACMG, American College of Medical Genetics and Genomics; LP, likely pathogenic; P, pathogenic; VUS variant or unkown significance; SDS: standard deviation score; dbSNP: Single Nucleotide Polymorphism Database; CADD: Combined Annotation Dependent Depletion; REVEL: Rare Exome Variant Ensemble Learner; GH: Growth Hormone; ACTH: Adrenocorticotropic hormone; TSH: Thyroid-Stimulating Hormone; LH: Luteinizing hormone; FSH: Follicle-stimulating hormone; PRL: Prolactin; ADH: Antidiuretic Hormone; F: Female; M: Male; SOD: Septo-optic dysplasia.

Table 3. Validated variants found in less frequently reported genes

Case	12	13	14	15	16	17
Age at time of diagnosis (years unless specified)	7	1	9	6 months	17	10
Gender	F	F	M	F	M	F
Hormone deficiencies	GH	GH TSH	GH ACTH TSH PRL LH FSH	GH TSH ACTH	GH TSH ACTH LH FSH	GH TSH LH FSH
Height SDS	-2.14	-4.72	-1.24	-5.98	-5.83	-1.6
Pituitary imaging						
Anterior	Hypoplasia	Hypoplasia	Hypoplasia	Hypoplasia	Hypoplasia	Normal
Stalk	Normal	Absent	Agenesia	Normal	Absent	Normal
Posterior	Normal	Orthotopic	Ectopic	Normal	Ectopic	Normal
Others	–	Enlarged subarachnoid spaces in the temporal lobe	–	–	Optic chiasm asymmetry	–
Associated malformations and features	Mammary hypertelorism, cubitus valgus and digital anomalies.	Mucocele, peculiar facies, hypoplasia of nares, depressed nasal bridge, neurodevelopmental delay and asymptomatic hypoglycaemia	Mild speech retardation, micropenis, microochidism, neonatal cholestasis and hypoglycaemia	Depressed nasal bridge,Frontal bossing,midfacial hypoplasia, SGA (height), neonatal jaundice	SGA, hypogonadotrophic hypogonadism	Chorioretinitis and retinitis pigmentosa, neurodevelopmental delay, and erythroblastopenia
Gene	*FOXA2*	*FOXA2*	*BMP4*	*FGFR1*	*PROKR2*	*PNPLA6*
Position (GRCh37)	20:22563198G>T	20:22563194G>T	14:54417042G>A	8:38271289A>G	20:5283150C>T	19:7622086A>C
Transcript	ENST00000419308	ENST00000419308	ENST00000245451	ENST00000425967	ENST00000217270	ENST00000450331
Variant	c.682C>A	c.686C>A	c.935C>T	c.2419T>C	c.691G>A	c.3343A>C
Protein	p.Arg228Ser	p.Ser229*	p.Ser312Leu	p.Tyr807His	p.Glu231Lys	p.Thr1115Pro
Type of mutation	Missense	Stop gained	Missense	Missense	Missense	Missense
Genotype	Heterozygous	Heterozygous	Heterozygous	Heterozygous	Heterozygous	Heterozygous
dbSNP	–	–	rs778093557	–	rs538606142	–
Polyphen	Probably damaging	–	Benign	Probably damaging	Probably damaging	Probably damaging
SIFT	Damaging	–	Damaging	Damaging	Damaging	Damaging
Mutation taster	Disease_causing	Disease_causing	Disease_causing	Disease_causing	Disease_causing	Disease_causing
CADD	26.7	39	23.1	27	23.2	24.7
REVEL	0.902	–	0.274	0.621	0.517	0.799
Allele frequency Gnomad	Absent	Absent	0.0000199 (0 hom)	Absent	0.000007954	Absent
Score ACMG	LP	LP	VUS	LP	P	LP
Score ACMG	PM2, PM1, PP3	PM1, PM2, PP3	BP1, BS2, PM1, PP3	PM2, PP2, PP3	BP1, PM2, PS3, PP3, PP4	PM1, PM2, PP3
Family history	Not tested	Not tested	Absent in healthy mother, father not tested	Absent in healthy father, mother not tested	Mother with short stature not tested	Present in the healthy mother, absent in the father

Abbreviations: ACMG, American College of Medical Genetics; CADD, Combined Annotation Dependent Depletion; dbSNP, Single Nucleotide Polymorphism Database; F, Female; LP, likely pathogenic; M, Male; REVEL, Rare Exome Variant Ensemble Learner; SDS, standard deviation score; SGA, Small for gestational age; SIFT, Sorting Intolerant from Tolerant.

Table 4. Validated variants fo und in new candidate genes

Case	18	19	20	21	22
Age at time of diagnosis (years unless specified)	1	6	5	1	6 months
Gender	F	F	M	M	F
Hormone deficiencies	GH TSH	GH	GH TSH ACTH	GH	TSH ACTH ADH
Height SDS	-2.43	-2.92	-1.57	-3.88	0.06
Pituitary imaging					
Anterior	Normal	Normal	Hypoplasia	Normal	Normal
Stalk	Normal	Normal	Normal	Normal l	Normal
Posterior	Normal	Normal	Ectopic	Absent	Absent
Others	-	-	-	Hypoplasia of the Sella turcica	corpus callosum and septum pellucidum agenesis, optic chiasm hypoplasia
Associated malformations and features	Low set ears, ulna valgus	–	Seizures and hypoglycaemia	Frontal bossing, depressed nasal bridge and small feet.	SOD. Hypoglycemia and neonatal jaundice.
Gene	*BMP2*	*HMGA2*	*HNF1A*	*HNF1A*	*NKX2-1*
Position (GRCh37)	20:6759134G>C	12:66232323C>T	12:121432115G>A	12:121426830C>T	14:36988556T>G
Transcript	ENST00000378827	ENST00000536545	ENST00000541395	ENST00000541395	ENST00000354822
Variant	c.589G>C	c.223C>T	c.862G>A	c.521C>T	c.97A>C
Protein	p.Val197Leu	p.Arg75Trp	p.Gly288Arg	p.Ala174Val	p.Met33Leu
Type of mutation	Missense	Missense	Missense	Missense	Missense
Genotype	Heterozygous	Heterozygous	Heterozygous	Heterozygous	Heterozygous
dbSNP	rs180897623	–	rs539507291	rs201934320	–
Polyphen	Probably damaging	Probably damaging	Probably damaging	Benign	Benign
SIFT	Damaging	Damaging	Damaging	Tolerated	Tolerated
Mutation taster	Disease_causing	Disease_causing	Disease_causing	Disease_causing	Disease_causing
CADD	25.5	35	23	26	22.3
REVEL	0.525	0.201	0.787	0.649	0.291
Allele frequency Gnomad	0.00000797 (0 hom)	absent	0.0000461 (0 hom)	0.0002 (0 hom)	0.000004087 (0 hom)
Score ACMG	LP	VUS	VUS	VUS	VUS
	BP1, PM1, PM2, PP3	PM1, PM2, PP3	BS2, PM1, PP2, PP3	BS1, BS2, PS2, PP2, PP3	BP4, PM2, PP3
Family history	Not tested	Not tested	Absent in healthy mother, father not tested	Absent in healthy parents	Not tested

Abbreviations: ACMG, American College of Medical Genetics; CADD, Combined Annotation Dependent Depletion; dbSNP, Single Nucleotide Polymorphism Database; LP, likely pathogenic; REVEL, Rare Exome Variant Ensemble Learner; SDS, standard deviation score; SIFT, Sorting Intolerant from Tolerant; VUS, variant of unknown significance.

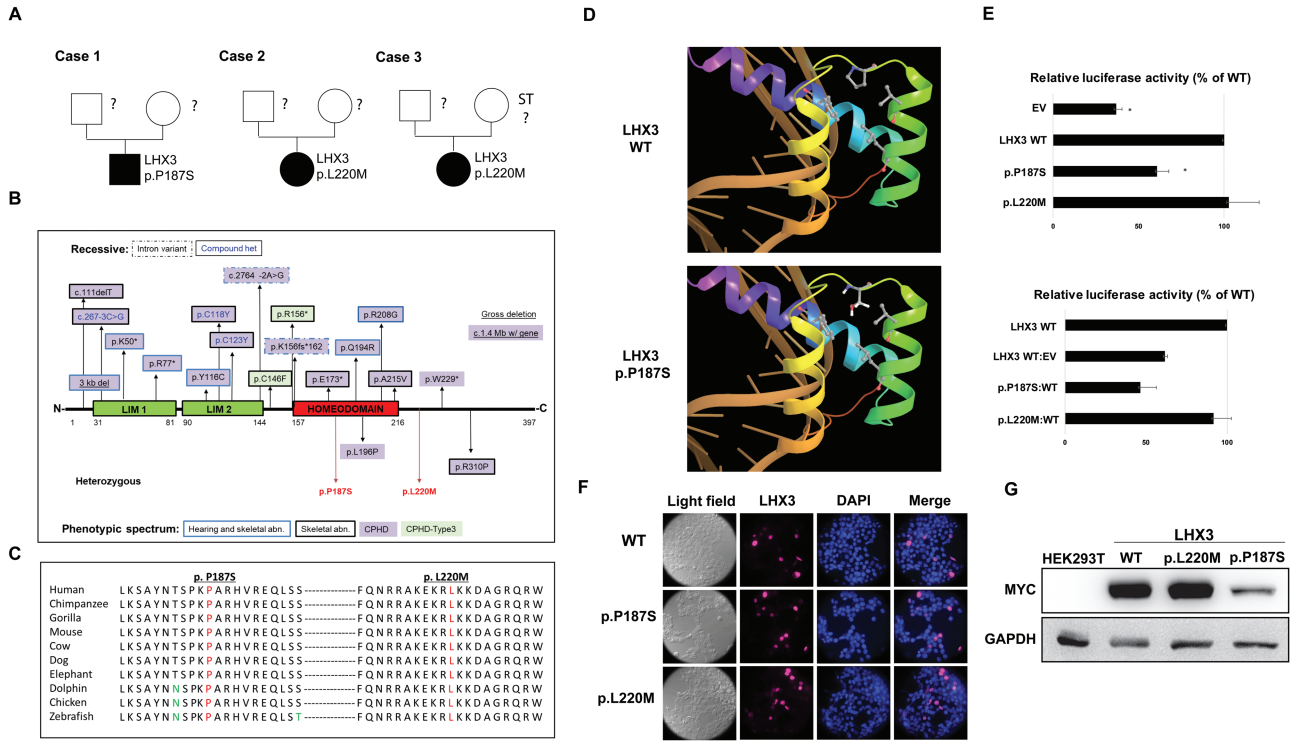

Figure 2. In silico and functional in vitro analysis of the *LHX3* identified variants. **A:** Pedigree of the three *LHX3* cases. Filled symbols represent patients with pituitary deficits, empty symbols indicate healthy individuals and question marks correspond to undetermined genotypes or DNA unavailable for testing. **B:** LHX3 protein structure and variants related to CPHD and other associated phenotypes. The variants from our cohort are heterozygous and shown in red. **C:** Amino acid conservation of LHX3 sequence between different species. Red indicates an evolutionary conserved residue whilst green shows an amino acid change. **D:** LHX3 protein structure cartoon showing the WT protein (upper panel) and p.Pro187Ser variant (bottom panel). Pro187 resides in the loop of a helix-turn-helix, and its replacement may alter the protein homeodomain and impair DNA binding. **E:** Functional transcriptional activation assays of the LHX3 variants using constructs bearing the amino acid changes and the human α-GSU promoter coupled to luciferase. HEK293T cell line was transfected with WT LHX3 or the variants to mimic a homozygous scenario (upper panel) or cotransfected with equal amounts WT and each indicated *LHX3* variant cDNA plasmid, to imitate a heterozygous state. The results are shown relative to the WT condition. Data shown are the mean ± SD of 3 independent experiments, each performed in triplicate. $*P < 0.05$ compared with WT. **F:** Immunofluorescence of the HEK293T cell line transfected with WT LHX3 or the variants. DAPI was used to visualize the nuclei. **G:** Evaluation of LHX3 expression in protein extracts from HEK293T cells transfected with WT LHX3 or LHX3 variants by Western blot using an anti-myc tag antibody. GAPDH was used as a loading control. Abbreviations: cDNA, complementary DNA; DAPI, 4′,6-diamidino-2-phenylindole; DNA, Deoxyribonucleic acid; EV, empty vector; GAPDH, Glyceraldehyde 3-phosphate dehydrogenase; SD, standard deviation; ST, short stature; WT, wild type.

normal and variant LHX3 proteins are translocated to the nucleus (Fig. 2F). Western blot revealed bands of the protein expected size for WT and variant LHX3, but protein levels of the variant LHX3 p.Pro187Ser are reduced compared to WT, suggestive of reduced stability (Fig. 2G).

Thus, we identified one likely damaging heterozygous *LHX3* variant in a patient with CPHD. Previously reported cases of CH caused by *LHX3* variants were recessive and sometimes included deafness or reduced neck rotation, which were absent in patient 1. Only 2 heterozygous variants were identified in CPHD patients that have a mild phenotype (30). This suggests that either this heterozygous p.Pro187Ser variant causes a mild phenotype or that other genetic or environmental factors likely contribute to the disease (30).

LHX4

Heterozygous mutations in this gene have been reported in patients with IGHD or CPHD, ectopic posterior pituitary (EPP), anterior pituitary (AP) hypoplasia, poorly developed sella turcica, and/or a Chiari malformation. Genetic screening led to the identification of 3 variants in this gene in unrelated families (Table 2). The patient from case 4 was diagnosed with short stature at the age of 2 years and had deficits in GH, TSH and ACTH, and SOD. He was heterozygous for an *LHX4*:c.300G>T, p.Gln100His missense variant in the LIM2 protein interaction domain. Patient 5 had CPHD involving the somatotroph, thyrotroph, lactotroph, and corticotroph axes and was heterozygous for *LHX4*:c.611G>T,p.Trp204Leu, a LP allelic variant in the homeodomain. He also carries a VUS c.895G>A,p.Ala299Thr in *FGFR1* (31). Patient 6 had GH and TSH deficiency, and carries a VUS variant *LHX4*:c.251G>A,p.Arg84His in the heterozygous state located in the LIM1 domain inherited from his healthy mother and a *GLI2* variant (c.3485A>G, p.Lys1162Arg) inherited from his healthy father (Fig. 3A–3B).

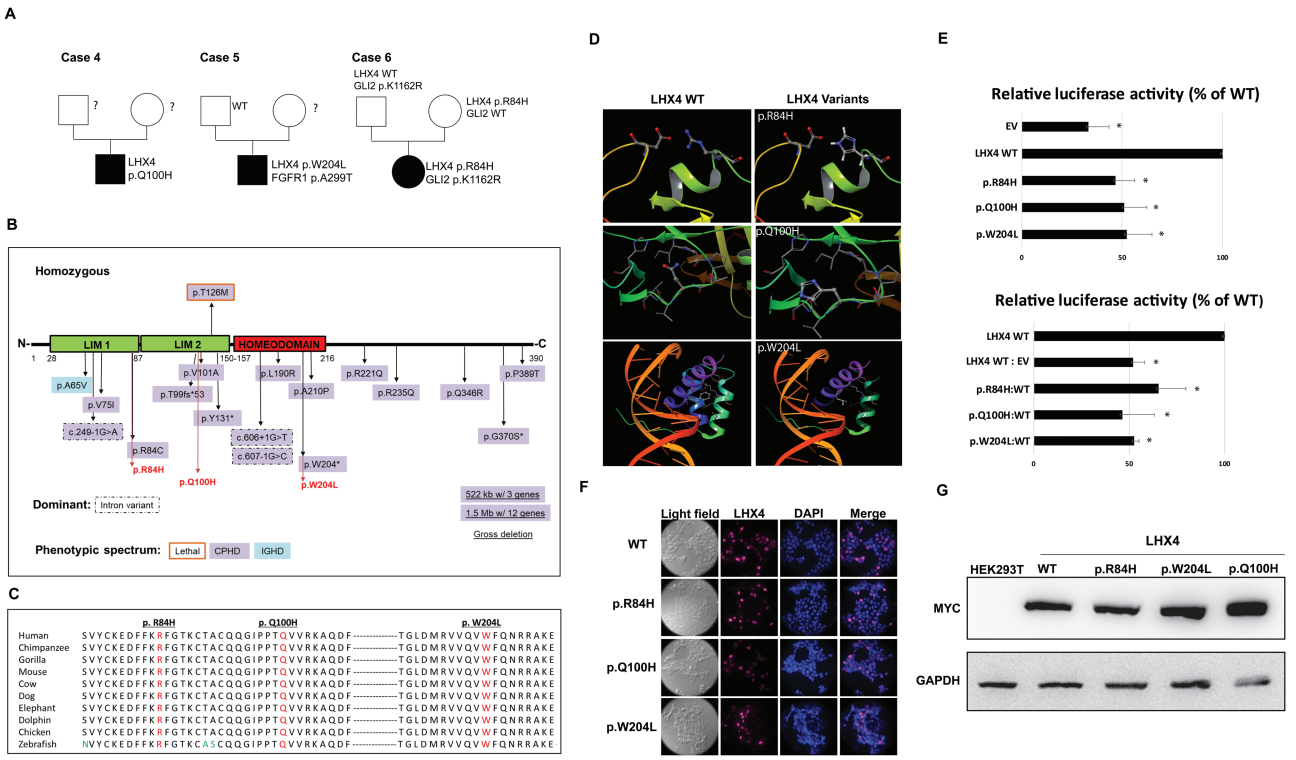

Figure 3. Identified *LHX4* variants, in silico analysis and functional in vitro assessment. **A:** Pedigree and genotype of the 3 families from our cohort which presented *LHX4* variants. Filled symbols represent patients with pituitary deficits, empty symbols indicate healthy individuals and question marks correspond to undetermined genotypes. **B:** LHX4 protein structure and variants related to IGHD or CPHD and other associated phenotypes. The variants from our cohort are heterozygous and shown in red. **C:** Amino acid conservation of LHX4 sequence between different species. Red indicates an evolutionary conserved residue whilst green shows an amino acid change. (**D**) LHX4 protein structure cartoon showing the WT protein and the selected variants. These models suggest that amino acid changes in p.Arg84His and p.Gln100His variants might affect structural conformation. Tryptophan at position 204 in LHX4 is part of a hydrophobic core, maintaining the interaction between the helix conformation of the homeodomain. Mutation to a small Leu might disrupt the structure affecting protein function. **E:** Functional transcriptional activation assays of the *LHX4* variants using constructs bearing the amino acid changes and the human GH promoter coupled to luciferase. HEK293T cell line was transfected with WT LHX4 or the variants to mimic a homozygous scenario (upper panel) or co-transfected with the same amount of WT *LHX4* and the indicated variant plasmids. The results are shown relative to the WT condition. Data shown are the mean ± SD of 3 independent experiments, each performed in triplicate. $*P < 0.05$ compared with WT. **F:** Immunofluorescence of the HEK293T cell line transfected with WT *LHX4* or the variants. DAPI was used to visualize the nuclei. **G:** Evaluation of LHX4 expression in protein extracts from HEK293T cells transfected with WT *LHX4* or *LHX4* variants by Western blot using an anti-myc tag antibody. GAPDH was used as a loading control. Abbreviations: CPHD, Combined Pituitary Hormone Deficiency; DAPI, 4′,6-diamidino-2-phenylindole; EV, empty vector; IGHD, Isolated Growth Hormone Deficiency; SD, standard deviation; WT, Wild type.

Multiple sequence alignment shows that the 3 LHX4 variants occur in highly conserved amino acids (Fig. 3C). Protein modeling suggests that the LIM domain variants, p.Arg84His and p.Gln100His, affect conformation. LHX4 p.Trp204Leu is part of a hydrophobic core that is essential for the homeodomain helix, and the smaller leucine residue could disrupt the structure (Fig. 3D). We tested the ability of *LHX4* variants to activate the proximal promoter of the human *GH1* gene (32, 33). The p.Arg84His, p.Gln100His, and p.Trp204Leu variants had reduced transactivation relative to WT (Fig. 3E). A different mutation at one of these residues, p.Arg84Cys, was previously reported in CH and was shown to have a transactivation defect (34). We assayed transactivation of the *GH1* reporter gene by a mixture of WT and mutant LHX4 proteins, simulating heterozygosity. We excluded a dominant negative effect of these variants over the WT LHX4 protein. Wild type and all LHX4 variants had similar nuclear localization (Fig. 3F). Western blotting of transfected cell extracts detected each LHX4 variant at the expected size and similar abundance (Fig. 3G). In summary, we identified 3 variants in *LHX4*, p.Arg84His, p.Gln100His, and p.Trp204Leu that are predicted to be damaging by at least 3 software programs, and had altered functional activity of the protein in vitro. These variants may explain the patients' CPHD features, as haploinsufficiency for *LHX4* has been reported in other patients. The SOD present in patient 4 suggests a multigenic disease with LHX4 contributing to the pituitary phenotype.

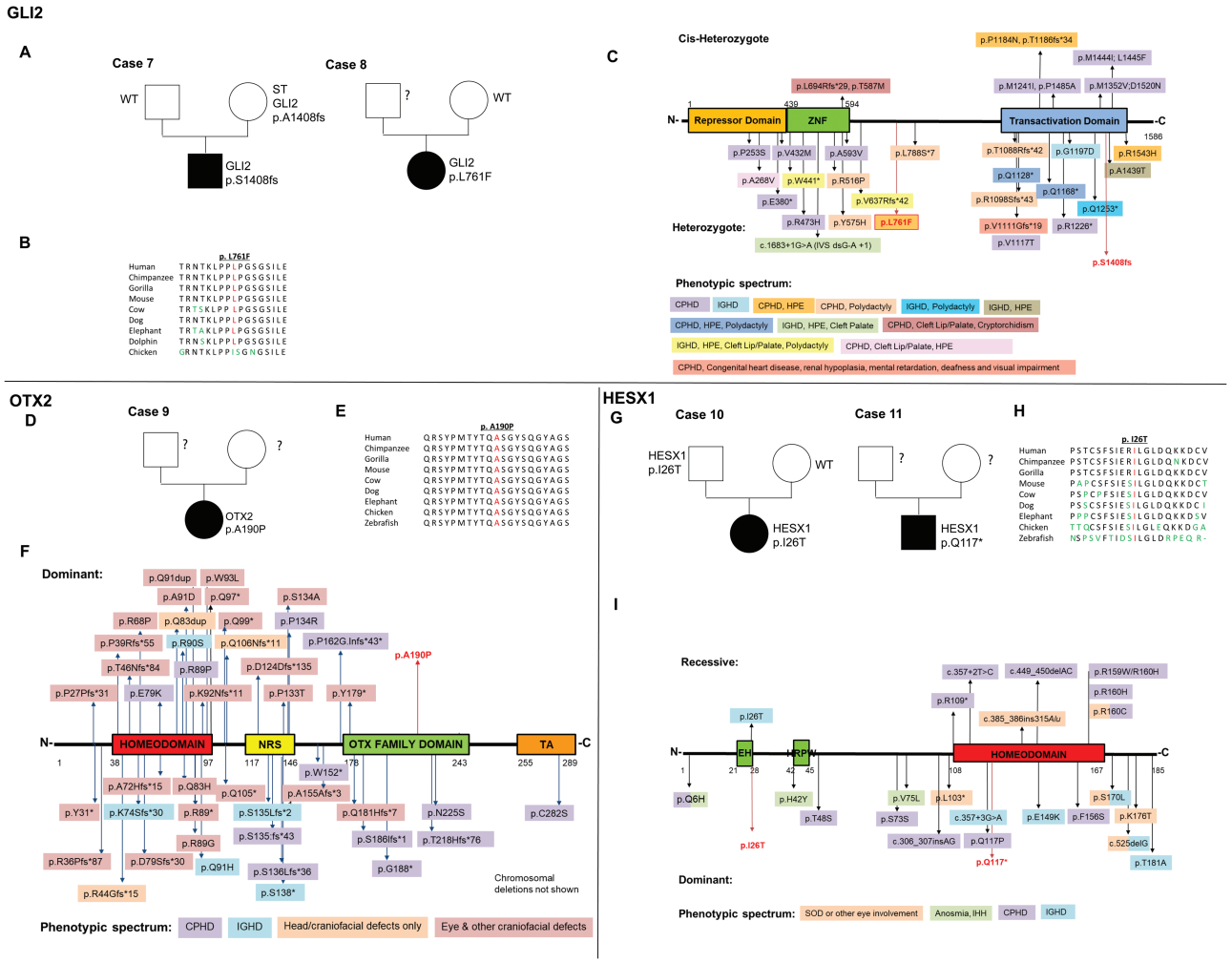

Figure 4. Identified variants in GLI2, OTX2 and HESX1. **A:** Pedigree of the two GLI2 variants. **B:** Amino acid conservation of GLI2 sequence between different species. Red indicates an evolutionary conserved residue whilst green shows an amino acid change. **C:** GLI2 protein structure and variants previously reported in relation to CPHD or IGHD and associated phenotypes. The variants from our cohort are heterozygous and shown in red. **D:** Pedigree of the variant found in the *OTX2*. **E:** Amino acid conservation of OTX2 sequence between different species. Red indicates an evolutionary conserved residue whilst green shows an amino acid change. **F:** OTX2 protein structure and variants related to CPHD, IGHD, and other pathologic phenotypes. The variants from our cohort are heterozygous and shown in red. **G:** Pedigree of the two HESX1 variants. **H:** Amino acid conservation of HESX1 sequence between different species. Red indicates an evolutionary conserved residue whilst green shows an amino acid change. **I:** HESX1 protein structure and variants related to IGHD, CPHD, and other associated phenotypes. The variants from our cohort are heterozygous and shown in red. In all pedigrees, filled symbols represent patients with pituitary deficits, empty symbols indicate healthy individuals and question marks correspond to undetermined genotypes/DNA not available for testing. Abbreviations: CPHD, Combined Pituitary Hormone Deficiency; DNA, Deoxyribonucleic acid; IGHD, isolated growth hormone deficiency.

GLI2

Patients with *GLI2* mutations have a range of clinical phenotypes, from CPHD to HPE, with severe defects in midline craniofacial development (35) (Fig. 4). Incomplete or variable penetrance was observed with mutations in this gene (36). We found 2 heterozygous variants in the *GLI2* gene in separate sporadic cases, and we expect them to be functionally significant. Patient 7 has GH and TSH deficiencies and a heterozygous duplication in the 3' portion of the *GLI2* gene (c.4207_4220dupATGGCTGCCATGCC), leading to a C-terminal frameshift (p.Ser1408TrpfsTer127). The mother had short stature (SDS–2.5) and the same LP duplication, which is predicted to be disruptive and absent in healthy control databases. Patient 8 had neonatal ADH deficiency and later developed GHD. She was heterozygous for *GLI2*:c.2281C>T, p.Leu761Phe, which is located between the DNA binding and transactivation domains. It was reported in a patient with SOD and CPHD and their healthy mother (37), although no functional analyses were performed. We hypothesize that this amino acid change could affect the acetylation of the nearby 757 lysine by modifying the recognition site of p300 acetylase. Acetylation of GLI2 at lysine 757 functions as a critical regulated step, controlling the activation status of Hedgehog pathway, one

of the major networks that regulates the key events during embryonic development (38).

OTX2

Patients with de novo or heterozygous *OTX2* mutations manifest highly variable phenotypes with incomplete penetrance, usually with ocular defects that may be associated with IGHD, CPHD, or hypogonadotropic hypogonadism (HH). We found a novel heterozygous missense mutation in the OTX protein–protein interaction motif: *OTX2*:c.568G>C, p.Ala190Pro in Case 9. This position is highly conserved from humans to zebrafish in all the homologous proteins tested (Fig. 4E). The amino acid change is potentially highly disruptive, as proline usually produces stiff turns that can alter protein structure. This variant may be sufficient to explain the patient's hypopituitarism phenotype.

HESX1

Several homozygous and heterozygous *HESX1* mutations have been reported in cases with IGHD, CPHD, and, occasionally, SOD and pituitary malformations (38–43). Patient 10 presented with IGHD and a heterozygous variant *HESX1*:c.77T>C,p.Ile26Thr. The same variant caused recessive CPHD with pituitary malformation in two unrelated cases (44).

Patient 11 presented with IGHD and thalassemia minor. He had a novel heterozygous stop codon variant c.349C>T, p.Gln117* in *HESX1*, which was classified as pathogenic, as it truncates the protein in the homeodomain, similar to previously reported truncations: p.Leu103* and p.Arg109* (4) (Fig. 4G–4I). This can explain the IGHD of this patient.

Variants in Less Frequently Reported Genes in CPHD Cases: *FOXA2, BMP4, FGFR1, PROKR2,* and *PNPLA6*

De novo heterozygous mutations in *FOXA2* (*600288) were reported in 5 patients so far, in individuals with CPHD, and occasional extrapituitary phenotypes, including hyperinsulinism (45, 46) or gastrointestinal malformations (47). We found rare, LP *FOXA2* variants in 2 patients with CPHD. Patient 12 had GHD and a heterozygous variant *FOXA2*:c.682C>A, p.Arg228Ser, predicted to be pathogenic (Fig. 5). Based on structural modeling, Arg228 likely interacts with the oxygens from the backbone of the loop (and the amino acid substitution likely abrogates that) destabilizing the local structure (Fig. 5D).

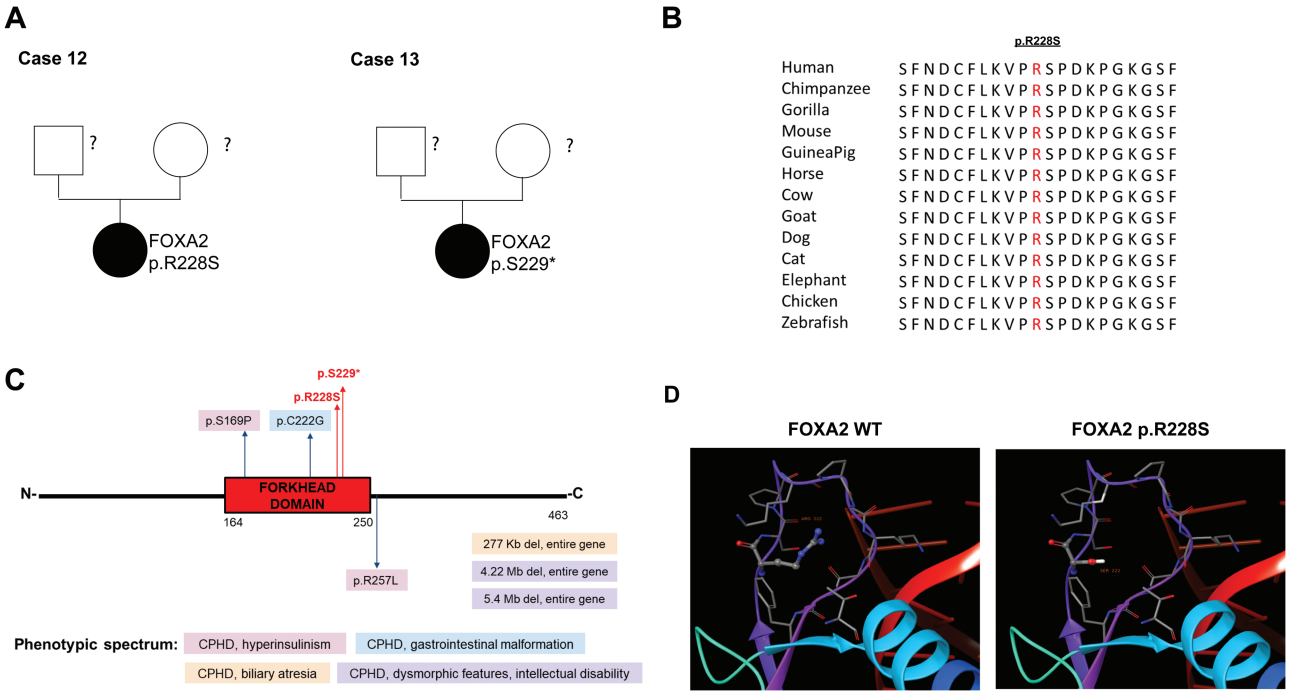

Figure 5. Identified variants in *FOXA2* and in silico analysis. **A:** Pedigree of the two *FOXA2* variants. Filled symbols represent patients with pituitary deficits, empty symbols indicate healthy individuals and question marks correspond to undetermined genotypes. **B:** Amino acid conservation of FOXA2 sequence between different species. Red indicates an evolutionary conserved residue whilst green shows an amino acid change. **C:** FOXA2 protein structure and variants previously reported in relation to CPHD. The variants from our cohort are heterozygous and shown in red. **D:** FOXA2 protein structure cartoon showing the WT protein (left panel) and p.Arg228Ser variant (right panel). It is possible that Arg in position 228 interacts with the oxygens from the backbone of the loop, which has a Lys inserted into de DNA indicating a potential role in said interaction. The mentioned amino acid change eliminates the possible interaction with the backbone in addition to the destabilization of the local structure. Abbreviations: CPHD, Combined Pituitary Hormone Deficiency; WT, wild type.

Patient 13 was born premature at 35 weeks of gestation and 10 days after birth had surgical removal of mucocele. She stayed hospitalized in neonatology with nasogastric tube feeding and mechanical ventilation for respiratory distress. Clinical investigation revealed asymptomatic hypoglycemia and GH and TSH deficiencies. She had a novel, stop codon mutation *FOXA2*:c.686C>A, p.Ser229* and an heterozygous *GLI1* (*165220) VUS (c.3142G>A, p.Asp1048Asn).

Both *FOXA2* variants are located within the forkhead domain, which may affect the DNA binding ability. Recent in vitro studies demonstrated functional impairment of two *FOXA2* variants (p.Arg257Leu and p.Pro169Ser) associated with hypopituitarism and impaired pancreatic β cell function (45, 46). Thus, we propose that the p.Arg228Ser and p.Ser229* variants are likely damaging based on the literature, the in silico prediction, and their absence in gnomAD and in-house population databases.

BMP4 (*112262) plays a central role in early patterning and growth of Rathke's Pouch (48). Human *BMP4* mutations are associated with brain, eye, and digital anomalies, and *BMP4* is involved in the etiology of CPHD (49). Five patients with CPHD and pituitary anomalies have been described carrying missense variants or deletions in the *BMP4* gene (50, 51). Here we report a missense variant *BMP4*:c.935C>T; p.Ser312Leu in patient 14 with CPHD (GH, TSH and ACTH deficiencies, low FSH/LH levels at mini puberty, and slightly elevated PRL levels). This variant is localized within the TGFβ-like domain like 2 other reported variants (p.Arg300Pro and p.Ala334Asp) identified in patients with CPHD and stalk anomalies (51, 52). Since the TGFβ-like domain has a key role in the protein function and in silico predictions argue for the pathogenicity of this variant, we hypothesize it explains patient's phenotype.

FGFR1 (*136350), is a tyrosine kinase receptor of the fibroblast growth factor receptor family and heterozygous variants can cause HH with or without anosmia, in combination with heterozygous loss of function variants in other genes. Fukami et al (53) was the first to report a heterozygous deletion in *FGFR1* in a patient with CPHD and neurological abnormalities. Patient 15 was deficient in GH, TSH, and ACTH, and heterozygous for *FGFR1*:c.2419T>C, p.Tyr807His. This novel variant was predicted to be damaging and absent in the control population. The patient may have variations in other genes that could have acted additively or synergistically with the *FGFR1* variant to cause the distinct CPHD phenotype.

PROKR2 (*607123) was initially identified as the causative gene of idiopathic hypogonadotropic hypogonadism and KS (54, 55). Several studies implicated heterozygous *PROKR2* variants in the pathogenesis of a wide spectrum of hypothalamo-pituitary disorders ranging from IGHD to CPHD (56), SOD (57) and Pituitary Stalk Interruption Syndrome (PSIS) (58). The patient from case 16 had deficits in GH, TSH, ACTH, LH, and FSH and a heterozygous variant *PROKR2*:c.691G>A, p.Glu231Lys within the transmembrane domain of the protein. The same heterozygous variant was found in a KS patient and interfered with PROKR2 signaling. Cells transfected with *PROKR2* p.Glu231Lys variant express 25% less receptor than WT cells, and they fail to activate G-protein signaling pathways in the presence of PROK2 (55). HH is genetically heterogeneous and can be oligogenic, patients typically lack GnRH stimulation to produce pituitary gonadotropins and have an impaired sense of smell. Some individuals may have HH due to pathogenic variants in PROKR2 and may be GH deficient due to variants in another gene or genes, resulting in a diagnosis of CPHD. In support of this idea, this patient was also heterozygous for a variant in the *TGIF1* (*602630) gene c.90G>A, p.Trp30*. This nonsense variant is very rare (0.000382), and no homozygotes have been reported. In addition, loss of function variants in *TGIF1* that are associated with HPE are incompletely penetrant and/or exhibit variable expressivity (59).

We found a missense *PNPLA6* (*603197) variant in a patient with CPHD, retinitis pigmentosa, and neurodevelopmental delay. Patient 17 was diagnosed with CPHD and had a novel heterozygous variant *PNPLA6*:c.3343A>C, p.Thr1115Pro absent from population databases. This change is likely disruptive because it is in the patatin-like phospholipase domain, critical for the activity of the NTE and is in close proximity to the protein's catalytic pocket. *PNPLA6* variants that disrupt the patatin-like domain are most likely the cause of the disease phenotypes found in Oliver-McFarlane and Laurence-Moon syndromes (60, 61). Since the healthy mother also has this variant and most reported *PNPLA6* variants were recessive (60), we do not discard the possibility of undiscovered gene variants in our patient.

New Candidate Genes: *BMP2*, *HMGA2*, *HNF1A*, and *NKX2-1*

During pituitary organogenesis, *BMP2* (*112261) signals stimulate the initial induction of Rathke's pouch and specification of all cell lineages in the anterior lobe. Here, we present patient 18 with GH and TSH deficiencies, dysmorphic features, and the missense variant *BMP2*:c.589G>C, p.Val197Leu, predicted to be probably damaging, LP according to ACMG criteria and localized in the propeptide domain, in which 2 other heterozygous variants (p.Ser37Ala and p.Arg190Ser) were associated with hypopituitarism (51, 62).

HMGA2 (*600698) is part of a group of nonhistone chromosomal proteins that participate in a wide variety

of cellular processes. Homozygous knockout mutations in *Hmga2* are responsible for the pygmy phenotype in mice (63). A GWAS identified a polymorphism in *HMGA2* that is significantly associated with childhood and adult height in the general population, and a 12q14 microdeletion affecting *HMGA2* causes proportionate short stature (64). Patient 19 presented IGHD, short stature (-2.92 SDS) and no other clinical findings. We identified a novel heterozygous VUS *HMGA2*:c.223C>T, p.Arg75Trp. This missense variant affects the 3rd AT-hook motif, is absent in control population databases, and this position is highly conserved. Arginine to tryptophan mutations are usually highly disruptive. This variant has been reported last year in a familial case of short stature and IGHD with an affected mother and son, both heterozygous (65), supporting the idea of a role of *HMGA2* in the IGHD etiology.

HNF1A (*142410) is a homeodomain-containing transcription factor expressed in the gastrointestinal axis with a key role in maintaining glucose homeostasis (66). Mutations in the human *HNF1A* gene cause a particular form of diabetes mellitus termed maturity-onset diabetes of the young (67). A heterozygous mutation in *HNF1A* exon 4 (c.872dup) was reported in a patient with diabetes, renal dysplasia, and hypopituitarism (68). We found 2 cases with heterozygous variants in *HNF1A*. Patient 20 presented with combined GH, TSH, and ACTH deficiencies, neonatal hypoglycemia, and seizures. No dysmorphic features were noted. We identified a novel heterozygous variant *HNF1A*:c.862G>A,p.Gly288Arg, located in the transactivation domain, predicted to be damaging by in silico algorithms, and classified as a VUS.

Patient 21 is a boy with IGHD, and presents a heterozygous VUS *HNF1A*:c.521C>T,p.Ala174Val, and compound heterozygous variants *DUOX2*:c.3050A>G; p.Glu1017Gly and c.2852-2A>G; splice acceptor. *DUOX2* encodes the enzyme responsible for the generation of hydrogen peroxide in thyrocytes, a critical step in the synthesis of thyroid hormones. Compound heterozygous variants in *DUOX2* were described in patients with congenital hypothyroidism and thyroid ectopy (CHTE). In particular, p.Glu1017Gly was reported in a child with CHTE who also carried other *DUOX2* variants (p.Pro138Leu, p.His678Arg and p.Ser1067Leu) in the heterozygous state (69). However, functional testing of the p.Glu1017Gly variant was not conducted. *DUOX2* deficiency presents a wide spectrum, from goiter to lifelong euthyroidism, and clinical expression varies between individuals and over time, justifying periodic reevaluation of the need for LT4 replacement (70). Patient 21 was 1 year old at the time of diagnosis, based on this genetic study; suggestions on follow-up thyroid levels through life were done.

NKX2-1 (*600635) encodes for TTF-1, a thyroid nuclear factor that regulates the expression of thyroid-specific genes and hypothalamic development. Human *NKX2-1* mutations have been associated with a rare syndrome known as benign hereditary chorea (BHC). About 6.4% of BHC patients with *NKX2-1* mutations developed pituitary abnormalities, including pituitary cystic masses, empty sella turcica, and stalk duplication (71, 72).

Patient 22 had TSH, ACTH, and ADH deficits. Arginine testing for GH secretion excluded GHD (peak 15 ng/ml). MRI studies revealed corpus callosum and septum pellucidum agenesis, optic chiasm hypoplasia, and the absence of posterior pituitary. Based on these findings, she was diagnosed with SOD and CPHD, and was treated with vasopressin, L-T4, and hydrocortisone. We found a heterozygous VUS missense variant *NKX2-1*:c.97A>C; p.Met33Leu, predicted to be disease-causing by mutation taster. An important role for *Nkx2-1* in thyroid and pituitary organogenesis has been demonstrated; however, its involvement in the etiology of CPHD remains unknown. We can speculate on the pathogenicity of the identified variant based on its low frequency on public databases, in silico prediction and the reported cases associated with pituitary anomalies resembling our patient's phenotype.

Variants in *GH1* in IGHD Cases

Variant *GH1*:c.626G>A,p.Arg209His was found in 3 independent families, representing a variant we found with a probable founder effect (Cases 23, 24, and 25 from Table 5). We first reported this variant in a 3-generation pedigree in (21), which is Case 23 in the present study. The same pathogenic mutation has been described in several pedigrees and may interfere with the secretion of GH in a dominant manner (73). Patient 24 is a male with GH deficiency and no other clinical manifestations; MRI imaging revealed normal pituitary structures. Patient 25 is a 3-year-old female, small for gestational age, and diagnosed with IGHD.

We found another variant *GH1*:c.55C>T,p.Pro19Ser in patient 26, a 22-year-old male with IGHD and normal pituitary MRI. This variant represents a change of proline, a small rigid amino acid that accentuates turns in the three-dimensional structure of the protein, to a serine, a small nonpolar amino acid. This change is somewhat disruptive to the normal structure of the hormone. This variant was found in a heterozygous state, was predicted pathogenic by 3 software programs, and has a low frequency in gnomAD. We hypothesized that this amino acid substitution compromises the ability of the signal peptide to direct GH to the endoplasmic reticulum for post-translational processing and hence inhibits its secretion. This mechanism

Table 5. Variant s in GH1

Case	23	24	25	26
Age at time of diagnosis (years unless specified)	9	9	3	22
Gender	F	M	F	M
Hormone deficiencies	GH	GH	GH	GH
Height SDS	–	-3.2	-2.6	
Pituitary imaging				
Anterior	Hypoplasia	Normal	Hypoplasia	Normal
Stalk	Normal	Normal	Normal	Normal
Posterior	Normal	Normal	Normal	Normal
Associated malformations and features	–	–	SGA (lenght)	-
Gene	*GH1*	*GH1*	*GH1*	*GH1*
Position (GRCh37)	17:61994697C > T	17:61994697C > T	17:61994697C > T	17:61995822G > A
Transcript	ENST00000323322	ENST00000323322	ENST00000323322	ENST00000323322
Variant	c.626G > A	c.626G > A	c.626G > A	c.55C > T
Protein	p.Arg209His	p.Arg209His	p.Arg209His	p.Pro19Ser
Type of mutation	Missense	Missense	Missense	Missense
Genotype	Heterozygous	Heterozygous	Heterozygous	Heterozygous
dbSNP	rs137853223	rs137853223	rs137853223	rs376449159
Polyphen	Benign	Benign	Benign	Benign
SIFT	Damaging	Damaging	Damaging	Damaging, tolerated
Mutation taster	Disease_causing	Disease_causing	Disease_causing	Disease causing, polymorphism
CADD	23.6	23.6	23.6	13.92
REVEL	0.803	0.803	0.803	0.479
Allele frequency Gnomad	0.00003186 (0 hom)	0.00003186 (0 hom)	0.00003186 (0 hom)	0.0001521 (0 hom)
	P	P	P	VUS
Score ACMG	PS3, PS4, PM2, PP2, PP3, PP5	PS3, PS4, PM2, PP2, PP3, PP5	PS3, PS4, PM2, PP2, PP3, PP5	PP2, PP3
Family history	Familial case (21)	Not tested	Absent in healthy mother, father not tested	Not tested

Abbreviations: ACMG, American College of Medical Genetics; CADD, Combined Annotation Dependent Depletion; dbSNP, Single Nucleotide Polymorphism Database; P, pathogenic; REVEL, Rare Exome Variant Ensemble Learner; SDS, standard deviation score; SIFT, Sorting Intolerant from Tolerant; VUS, variant of unknown significance.

was demonstrated before for the GH1 p.Leu11Pro variant found in a severe case of IGHD (74).

Discussion

In this study, we screened 67 genes in a cohort of 170 patients with congenital pituitary deficiencies from Argentina using a gene panel based on smMIPS. Among these, 30 genes were previously implicated in CPHD, and the remainder were selected because of their role in related disorders, relationships with known genes, and/or implications from mouse studies. Thus, we had the opportunity to test for the frequency of mutations in known genes in this population and to discover novel genes that could contribute to disease. We obtained 700X average coverage per sample over targeted regions. This approach allowed us to identify relevant variants that were later confirmed by Sanger sequencing. To our knowledge this is the first study screening a cohort of this size and testing multiple genes in parallel for this endocrine disorder. Most of the published reports analyze single cases or a single patient, and/or multiple cases but with only a few genes tested (75–80). smMIPS approach is a highly sensitive and reliable technique to detect genetic variants, is rapid and cost-effective, requires low amounts of DNA and allows the processing of multiple samples in parallel.

In the present study, based on in silico analysis, 4 pathogenic and 7 likely pathogenic variants were identified in 15/170 patients analyzed. In the literature, the positive rate of genetic screening of CH is low (1.9–4% in sporadic cases) (81). Pathogenic variants were found in *GH1* (in 3 independent families), in *GLI2*, *HESX1*, and *PROKR2*. Thirteen candidate VUS variants were also identified. We evaluated the functional consequences of VUS in *LHX4* and *LHX3*. All of the LHX4 variants identified in our cohort (p.Arg84His,

p.Gln100His and p.Trp204Leu) were present in the heterozygous state and had reduced transactivation function. This is consistent with other reports of haploinsufficiency for *LHX4* variants in CPHD patients (32, 34). Of note, *LHX4* p.Trp204Leu and p.Arg84His were found in patients who also had additional variants in other genes. Thus, the mechanism of pathogenicity in these cases may be complex, involving a combination of variants in more than 1 gene (oligogenic inheritance) and de novo variants occurring in individual patients. Our experiments showed that LHX3:p.Pro187Ser variant, but not p.Leu220Met, significantly reduced protein function in vitro. Most cases with homozygous LHX3 variants reported in CPHD were associated with a more severe phenotype including ear defects or stalk interruption. Thus, the heterozygous LHX3 variants presented here may contribute to the mild phenotype within hypopituitarism spectrum disorder in our patients.

A novel aspect of the present work is the low frequency of *PROP1* mutations in our cohort. Mutations in *PROP1* are the most common, known cause of CPHD, accounting for 11% of total cases worldwide. *PROP1* mutations showed a conspicuously high frequency in East Central and Eastern European countries with a Baltic or Slavic ethnic background due to 2 founder events about 101 and 44 generations ago (7). The frequency of *PROP1* mutations varies widely by population group, and the rate was previously unknown for Argentina. Interestingly, no cases of pathogenic *PROP1* mutations were found in this study.

One important novelty from our study are LP variants found in novel genes in the etiology of CPHD, as *FOXA2*. De novo heterozygous mutations in *FOXA2* have been reported in 5 cases of CPHD and congenital hyperinsulinism with other features, including craniofacial dysmorphic features, choroidal coloboma, and endoderm-derived organ malformations (46). We found 2 variants within the forkhead domain of FOXA2, which may affect the DNA binding ability of the protein and are predicted to be damaging.

In conclusion, genetic causes of pituitary hormone deficiency can manifest with a range of clinical symptoms and variable penetrance, which provides a challenge for predicting phenotype from genotype. The novel mutations identified in this study provide new insights into the biology of the affected genes and proteins and the highly variable clinical phenotypes. Identifying potential pathogenic variants will make it feasible to predict clinical outcomes from genetic data, which is necessary for patient diagnosis and prognosis, and for assessing the risk of future affected individuals.

Conclusion

In this study we showed that the smMIPS approach is an economical and effective method to screen known and novel candidate genes for CH and related disorders. This method may also enable prioritization of "unexplained" cases for more comprehensive follow-up analyses. The benefits of a genetic diagnosis for patients and parents are manifold and include information for genetic counseling and state-of-the-art clinical management. We expect this work will provide a foundation for endocrinologists and other clinicians as they expand their work into precision medicine. We also believe that data sharing among researchers and clinicians is critical to improve genetic diagnosis of rare genetic endocrine disorders.

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