




Heterozygous LHX3 mutations may lead to a mild phenotype of combined pituitary hormone deficiency

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Abstract

LHX3 is an LIM domain transcription factor involved in the early steps of pituitary ontogenesis. We report here functional studies of three allelic variants, including the first heterozygous variant of *LHX3* NM_178138.5(LHX3):c.587T>C (p.(Leu196Pro)) that may be responsible for a milder phenotype of hypopituitarism. Our functional studies showed that NM_178138.5(LHX3):c.587T>C (p.(Leu196Pro)) was not able to activate target promoters in vitro, as it did not bind DNA, and likely affected LHX3 function via a mechanism of haplo-insufficiency. Our study demonstrates the possibility that patients with a heterozygous variant of *LHX3* may have pituitary deficiencies, with a milder phenotype than patients with homozygous variants. It is thus of vital to propose an optimal follow-up of such patients, who, until now, were considered as not being at risk of presenting pituitary deficiency. The second variant NM_178138.5(LHX3):c.622C>G (p.(Arg208Gly)), present in a homozygous state, displayed decreased transactivating ability without loss of binding capacity in vitro, concordant with in silico analysis; it should thus be considered to affect LHX3 function. In contrast, the NM_178138.5(LHX3):c.929G>C (p.(Arg310Pro)) variant, in a heterozygous state, also predicted as deleterious in silico, proved functionally active in vitro, and should thus still be classified as a variant of unknown significance. Our study emphasizes the need for functional studies due to the limits of software-based predictions of new variants, and the possible association of a pituitary phenotype to heterozygous *LHX3* variants.

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Introduction

Combined pituitary hormone deficiency (CPHD) is defined as the presence of constitutional deficiency of at least two anterior pituitary hormones, with or without associated syndromic features. Variants of genes involved in late pituitary development (such as *PROPI*, *POU1F1*, or *TBX19*) are associated with endocrine phenotypes without extra-pituitary defects [1], while variants of early-expressed

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genes, such as *LHX3*, *LHX4*, or *HESX1*, are commonly associated with extra-pituitary malformations including neck rotation abnormalities, hearing loss, or septo-optic dysplasia.

In mice, the normal expression of the LIM (LIN-11, Isl1, and MEC-3)-homeodomain transcription factor LHX3 is necessary for pituitary development and function [2–5]. In rodents, LHX3 is necessary for survival, since mice with homozygous inactivation of *Lhx3* (*Lhx3*^{-/-}) die soon after birth; their pituitaries are aplastic and lack the anterior and intermediate lobes [2, 6]. In contrast, *Lhx3*^{+/-} mice have normal pituitaries and no specific phenotype [7]. The overexpression of two isoforms of LHX3 (LHX3a or LHX3b) leads to a developmental defect of the gonadotroph axis [8]. LHX3a activates the transcription of pituitary genes such as *Alpha-Gsu*, *Prolactin*, *β-Tsh*, *β-Fsh*, and *GnRH receptor*. LHX3 is also required for the expression of early (HESX1, SF1) and late-acting transcription factors (POU1F1) involved in pituitary development. Finally, LHX3 and POU1F1 interact synergistically to activate the promoters of *β-Tsh* and *Prolactin* [9]. Overall, these data are in favor of a major role of LHX3 during pituitary development. Of note, LHX3 is also involved in the development of motor neurons and the inner ear [10, 11], which explains the complex phenotype of some patients carrying *LHX3* variants.

In humans, homozygous variants of *LHX3* lead to CPHD. To date, 14 autosomal recessive variants of *LHX3* have been reported in patients with CPHD [12–21]. All of these variants were identified in exons, except for one located in an intronic region [18]. Patients bearing *LHX3* variants displayed consistent somatotroph, thyrotroph and gonadotroph deficits, and inconsistent corticotroph deficits. On magnetic resonance imaging (MRI), the pituitary was described as hypoplastic or aplastic in 60% of cases, normal in 10%, and hyperplastic in 30% of cases. The pituitary phenotype was frequently associated with a rigid neck (70% of patients), vertebral anomalies and more or less severe hearing loss (50% of cases).

In this study, we report the first heterozygous variant of *LHX3* likely responsible for CPHD. We also report the results of functional studies of two newly identified variants of *LHX3* presumed to be deleterious in silico, one in a homozygous and the other in a heterozygous state.

Materials and methods

Subjects

GENHYPOPIT is a clinical research network that was launched to investigate the genetic basis of CPHD [22]. In this context, based on previous reports and our own

experience, genetic screening of *LHX3* variants (RefSeq: NM_178138.5; OMIM 600577) was performed specifically in patients with at least growth hormone (GH), thyrotropin-stimulating hormone (TSH), and luteinizing/follicle-stimulating hormone (LH/FSH) deficiencies. Patients with a known postnatal cause of acquired hypopituitarism were excluded. Hormonal studies and intracranial imaging were performed in all patients at each referring medical center. Malformations were systematically sought on MRI and recorded. Blood samples were collected from patients and, wherever possible, from first-degree relatives. Informed written consent was obtained from the parents, caretakers, or guardians on behalf of minors/children enrolled in the study. The study was approved by the Ethics committee of the University of Aix-Marseille (France).

Screening for *LHX3* variants

One hundred and sixty-four patients with features suggesting the presence of *LHX3* variants were screened. DNA was extracted from blood lymphocytes. Genomic DNA was PCR-amplified from all index cases using sets of flanking intronic primers for direct sequencing of all coding exons of *LHX3* (primer sequences available in Supplemental Data). Amplification was carried out using the Hot Start Taq polymerase kit protocol (Qiagen GmbH, Hilden, Germany). Sequencing was performed with a 3130 XL Genetic analyzer (LifeTechnologies, New York, USA). Sequences were analyzed using Variant Reporter software (LifeTechnologies, New York, USA).

Next-generation sequencing and multiplex ligation-dependent probe amplification (MLPA)

Genomic DNA was extracted from blood lymphocytes (standard EDTA samples) using QiaSymphony DS DNA Midi Kit (Qiagen, France). Exons and 20 bp flanking introns of 16 genes, confirmed as causal in combined pituitary hormones deficits (*FGF8*, *FGFR1*, *GHI*, *GLI2*, *HESX1*, *IGSF1*, *LHX3*, *LHX4*, *OTX2*, *PAX6*, *PROPI*, *POU1F1*, *PROKR2*, *SOX3*, *SOX2*, *TBX19*, and *ARNT2*), were amplified using a QIAseq Targeted DNA Custom Panel (Qiagen, France). The preparation of the library was completed according to the manufacturer's instructions. All sample preparations were then sequenced on MiseqDX (Miseq V2 Reagent Kit 300 cycles) (Illumina®, San Diego, CA, USA). Sequencing data were exported and further analyzed (base calling, read filtering, alignment to the human genome (GRCH37/hg19) and annotated by using Biomedical Genomic Workbench v4.1.1. software (Qiagen, France). Variant calling analysis was checked further on VariantStudio v2.2 (Illumina®, San Diego, CA, USA). Coverage, for a depth exceeding 30x, was 100% in the

targeted regions (16 samples in a run). All variants of interest were visually inspected in the Integrative Genomics Viewer (<https://www.broadinstitute.org/igv>) to evaluate mapping and variant calling quality. The candidate variants were then classified into five main categories using the American College of Medical Genetics and Genomics guidelines—pathogenic, likely pathogenic, variants of uncertain significance (VUS), likely benign, and benign [23]. Sanger sequencing was performed to validate those variants categorized as pathogenic, likely pathogenic, or VUS.

Multiplex ligation-dependent probe amplification (MLPA) assays were performed, as a partial check of next-generation sequencing (NGS) data to exclude gene copy number alteration (deletion and duplication) in the three patients, by using a commercially available MLPA kit (P216-B1), following the protocol provided by the manufacturer (MRC Holland, Amsterdam, The Netherlands). This MLPA kit includes probes for all coding exons of *LHX3* (both transcripts) and of five other genes, included in the NGS panel, and possibly involved in CPHD (*POU1F1*—except exon 5, *PROPI*, *HESX1*, *LHX4*, and *GHI*—except exons 1 and 2). MLPA products were run on ABI Prism 3500XL sequencer and analyzed using Coffalyser. Net software (MRC Holland, Amsterdam, The Netherlands).

In silico analysis

Alignment of amino acid sequences was performed using UniProtKB (www.uniprot.org). Polyphen 2 (<http://genetics.bwh.harvard.edu/pph2>), Predict SNP (<http://loschmidt.chemi.muni.cz/predictsnp>), and UMD-HTS (www.umd-hts.eu/WHTS9) were used for each allelic variant. Evaluation of variant frequency was performed with GnomAD. ACMG-AMP guidelines for the interpretation of sequence variants were used to determine the functional status of each variant.

Plasmid constructs and mutagenesis

The 550 bp *alpha-GSU* promoter (pGL3-*alphaGSU/Luc*) and the 1273 bp *beta-TSH* promoter (pGL3-*betaTSH-1236/Luc*) were provided by S. Amselem (Laboratoire de Génétique Médicale, Hôpital Trousseau, Paris, France). The reporter plasmid *hPRL-250/Luc*, containing 164 bp of the human PRL proximal promoter, was a gift from Dr. J. Martial (Liege University, Belgium). The human *LHX3a* cDNA, containing an myc tag in its-C-terminal part and inserted into the CMV-driven eukaryotic expression vector pcDNA3.1 (hereafter referred to as pcDNA) (Invitrogen), was provided by S. Rhodes (Indiana University School of Medicine, Indianapolis, USA).

Full-length human *POU1F1* cDNA was cloned by PCR, using normal pituitary tissues and inserted in the pcDNA expression vector (pcDNA-*POU1F1*). The NM_178138.5 (*LHX3*):c.587T > C (p.(Leu196Pro)), NM_178138.5 (*LHX3*):c.622C > G (p.(Arg208Gly)), and NM_178138.5 (*LHX3*):c.929G > C (p.(Arg310Pro)) variants in the pcDNA-*LHX3a-myc* construct, designated *LHX3-p*. (Leu196Pro), *LHX3-p*.(Arg208Gly), and *LHX3-p*.(Arg310Pro), respectively, in the text, were generated, with the QuickChange Mutagenesis Kit (Stratagene, La Jolla, CA, USA) and the following commercially synthesized oligonucleotides (IDT, Leuven, Belgium); variants are indicated in bold and underlined (only the sense strand oligonucleotide is shown):

NM_178138.5(*LHX3*):c.587T > C (p.(Leu196Pro)): 5'-CTCTCGTCCGAGACGGGCCCGGACATGCGCGTGTGC-3';

NM_178138.5(*LHX3*):c.622C > G (p.(Arg208Gly)): 5'-GCAGGTTTGGTTCCAGAACGGCCGGGCCAAGGAGAAGAGG-3';

NM_178138.5(*LHX3*):c.929G > C (p.(Arg310Pro)): 5'-GAGCAGTACCGAGAGCTGCCTCCCGGCAGCCCCTACGG-3'.

The wild-type pcDNA-*LHX3a-myc* construct is referred to as *LHX3-WT*.

Cell culture and transfections

HEK293T cells and COS-1 cells were grown in 12-well plates in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum. Cells were transfected in serum-free medium with Lipofectamine reagent (Invitrogen) according to the manufacturer's instructions. For the study of the transactivating abilities of *LHX3-WT* and mutants, the total amount of DNA (600 ng) was kept constant by cotransfection of pcDNA empty vector, and 400 ng promoter (*alpha-Gsu*, *TSHB* and prolactin) was transfected for each well. For the study of the dominant-negative effect of *LHX3-p*.(Leu196Pro), 150 ng of *LHX3-WT* was transfected with 150 ng of *LHX3-p*.(Leu196Pro) and compared to 300 ng of *LHX3-WT*. For the study of synergistic effects of *LHX3* and *POU1F1* on the prolactin promoter, 300 ng of *LHX3-WT* or mutant was transfected with 200 ng of *POU1F1* and 400 ng of the prolactin promoter. For all experiments, cotransfection with 0.01 ng pCMV-*Renilla* was performed.

Dual luciferase assay

HEK293T and COS-1 cells were prepared and assayed for luciferase activity 48 h after transfection, using the Dual-

luciferase Assay system (Promega). Transfection efficiency was determined using Renilla luciferase luminescence, and luciferase firefly values were normalized to it. Firefly and Renilla luciferase activity was measured 48 h after transfection. All assay points were derived in triplicate. All experiments were performed at least three times. Results are expressed in relative units (fold-increase vs empty vector).

Cell extracts

Forty-eight hours post transfection cells (HEK293T and COS-1) were detached by scraping in ice-cold PB5/5 mM EDTA. Total protein lysates were obtained by RIPA extraction followed by sonication. Nuclear and cytoplasmic proteins were extracted with Thermo Scientific NE-PER Nuclear and Cytoplasmic Extraction Reagents following the manual instructions. For both extractions, Roche complete™ protease inhibitor (1×) and sodium orthovanadate (1 mM) were used to prevent degradation and dephosphorylation. Protein content in the lysates was measured by colorimetric analysis using an improved Bradford assay (Pierce Coomassie Plus Reagent).

Western blot

HEK293T and COS-1 cells transfected with LHX3 expression vectors were analyzed by western blot. Five micrograms of protein extracts were run on a 10% acrylamide gel and transferred to a low fluorescence PVDF membrane (Millipore) using Pierce G2 Fast Blotter and 1-Step transfer buffer. The membrane was then incubated with mouse anti-Myc primary antibody (1/1000, clone 9E10 ThermoFisher Scientific) or anti-LHX3 hybridoma supernatant (clone 67.4E12, 1/200 DSHB, University of Iowa), followed by the secondary antibody (Alexa Fluor 647-coupled donkey anti-mouse monoclonal antibody diluted 1/1000; Invitrogen). ImageJ was used to perform relative quantification by a series of dilutions of LHX3-WT extracts for each membrane.

Electrophoretic mobility shift assay

Electrophoretic mobility shift assay (EMSA) experiments were performed using the previously described LUEGO technique (double-labeled Cy5 way) [24] with probes representing the pituitary glycoprotein basal element of the α GSU gene: 5'GGTACTTAGCTAATTAATGTG3'. Binding was done in {10 mM Tris-HCl, 5 mM DTT, 50 mM NaCl, 7.4 mM MgCl₂, 5% saccharose, 500 ng/μl BSA, 0.1% NP40} with 8.3 nM probe and approximately 2 μg protein extract for 10 min at room temperature. The exact quantity of the protein extract was determined to ensure that the same quantity of protein was analyzed for LHX3-WT

and all the mutants, as evaluated by quantitation of western blots. Supershift assay was performed with 0.5 μg of mouse anti-6xHis antibody (Thermo Scientific). Depending on the condition, unlabeled wild type (same sequence as labeled probe) or mutated (5'-GGTACTTAGCTAActgacTGTG-3') probes were used as competitors (50-fold higher concentration). Visualization was performed with a Typhoon FLA 9000 scanner (GE Healthcare).

Statistical analysis

Data points were compared using a one-tailed Student's *t*-test for paired samples using XLStat 2013.4.05 (Paris, France). *p* < 0.05 was considered statistically significant.

Results

Individual data of the patients bearing LHX3 variants

Pedigree A: NM_178138.5(LHX3):c.587T>C (p.(Leu196Pro)) allelic variant (heterozygous) (Chr9 (GRCh37):g.139090773A>G), submitted to LOVD (www.lovd.nl/LHX3, individual ID: 00163928) (see Table 1 for phenotypic details).

The proband was an Italian male with neonatal panhypopituitarism and a micropenis. Cerebral MRI appeared normal. The mother of the proband carried the same variant and had a normal pituitary evaluation up to age 40. The patient also presented with one *ARNT2* variant, five *GLI2* variants, three *IGSF1* variants, two *LHX3* variants, one *LHX4* variant, one *PROKR2* variant, and two *PROPI* variants, all considered benign or likely benign, according to the ACMG-AMP guidelines (Supplemental File 1).

Pedigree B: NM_178138.5(LHX3):c.622C>G (p.(Arg208Gly)) allelic variant (homozygous) (Chr9 (GRCh37):g.139090651G>C), submitted to LOVD (www.lovd.nl/LHX3, individual ID: 00163929)

The proband was an Iranian girl presenting with neonatal panhypopituitarism. The phenotype also included retinal dystrophy and low-set ears. Cerebral MRI showed a cystic anterior pituitary and corpus callosum digenesis. Her parents were consanguineous. No genetic analysis was available for any other family member. The patient also presented with five *GLI2* variants, four *IGSF1* variants, two *LHX3* variants, one *LHX4* variant, one *PAX6* variant, and four *PROPI* variants, all considered benign or likely benign, according to the ACMG-AMP guidelines (Supplemental File 1).

Pedigree C: NM_178138.5(LHX3):c.929G>C (p.(Arg310Pro)) allelic variant (heterozygous) (Chr9 (GRCh37):g.139089436C>G), submitted to LOVD (www.lovd.nl/LHX3, individual ID: 00163930).

Table 1 Phenotypic and biological profiles of the patients bearing the three variants of *LHX3*

Variant	NM_178138.5 c. 587T>C p.(Leu196Pro)	NM_178138.5 c. 587T>C p.(Leu196Pro)	NM_178138.5 c. 622C>G p.(Arg208Gly)	NM_178138.5 c. 929G>C p.(Arg310Pro)
Age at evaluation (years)	2	40	2	3
Status	Propositus A	Mother (Pedigree A)	Propositus B	Propositus C
Country	Italy	Italy	Iran	Argentina
Medical history of hypopituitarism in family	No	No	No	No
Zygoty	Heterozygous	Heterozygous	Homozygous	Heterozygous
ACTH deficiency	Yes (neonatal)	No	Yes (neonatal)	Yes (neonatal)
TSH deficiency	Yes (neonatal)	No	Yes (neonatal)	Yes (3 years)
FSH/LH deficiency	NE	No	NE	NE
GH deficiency	Yes (neonatal)	No	Yes (neonatal)	Yes (2 years)
Anterior pituitary on MRI	Normal	Normal	Hypoplastic with a cyst	Hypoplastic
Posterior pituitary MRI	Normal	Normal	Normal	Not visualized on MRI
Pituitary stalk MRI	Normal	Normal	Normal	Thin
Associated malformations	No	No	Low-set ears, retinal dystrophy, corpus callosum digenesis	Microcephaly

For pituitary deficiencies, data between brackets represent age at diagnosis. Complete GH deficiency was defined as GH response after stimulation below 10 mUI/L. Corticotroph deficiency was defined as plasma cortisol value below 500 nmol/L after insulin stimulation test. Gonadotroph axis was investigated only in patients of postpubertal age, i.e., older than 15 years for female and 17 years in male subjects (NE not evaluated). Thyrotroph deficiency was defined as low or normal basal TSH levels associated with low T4 levels. None of the patient was presenting with sensorineural hearing loss, vertebral anomalies, or neck rotation abnormalities

The propositus was a boy from Argentina with neonatal panhypopituitarism associated with microcephaly and a micropenis. He did not show signs of hypoglycemia at birth. Cerebral MRI showed anterior pituitary hypoplasia, a thin pituitary stalk, and a non-visualized posterior pituitary. No genetic analysis was available for any other family member. The patient also presented with five *GLI2* variants, one *HESX1* variant, five *IGSF1*, two *LHX3* variants, two *LHX4* variant, one *PROKR2* variant, and three *PROPI* variants, all considered benign or likely benign, according to the ACMG-AMP guidelines (Supplemental File 1).

In silico analyses

All of the *LHX3* amino acid residues modified by the allelic variations observed in our three index patients are conserved in known *Lhx3* sequences in mammals, mice, chicken, and fish (data not shown). Software-based predictions suggested that the three allelic variants would be presumed to alter the function of the protein. In GnomAD, NM_178138.5(*LHX3*):c.929G>C (p.(Arg310Pro)) variant

was reported with a population frequency of 0.064% in a heterozygous state, while the two other variants had never been reported. NM_178138.5(*LHX3*):c.929G>C (p.(Arg310Pro)) variant was considered as a variant of unknown significance in dbSNP and ClinVar. These three variants were designated VUS, according to the ACMG-AMP guidelines for the interpretation of sequence variants, prior to functional studies.

In vitro analyses

Western blot

We evaluated the expression of WT and mutant *LHX3* proteins by western blot using cytoplasmic and nuclear extracts from transfected HEK293T cells. Bands of the expected size for *LHX3*-WT, *LHX3*-p.(Leu196Pro), *LHX3*-p.(Arg208Gly), and *LHX3*-p.(Arg310Pro) were detected. Of note, a high level of *LHX3*-p.(Leu196Pro) was found in the cytoplasm of transfected cells (and this was not observed for *LHX3*-WT and the other mutants) (Fig. 1a). The same result was observed when the western blot was

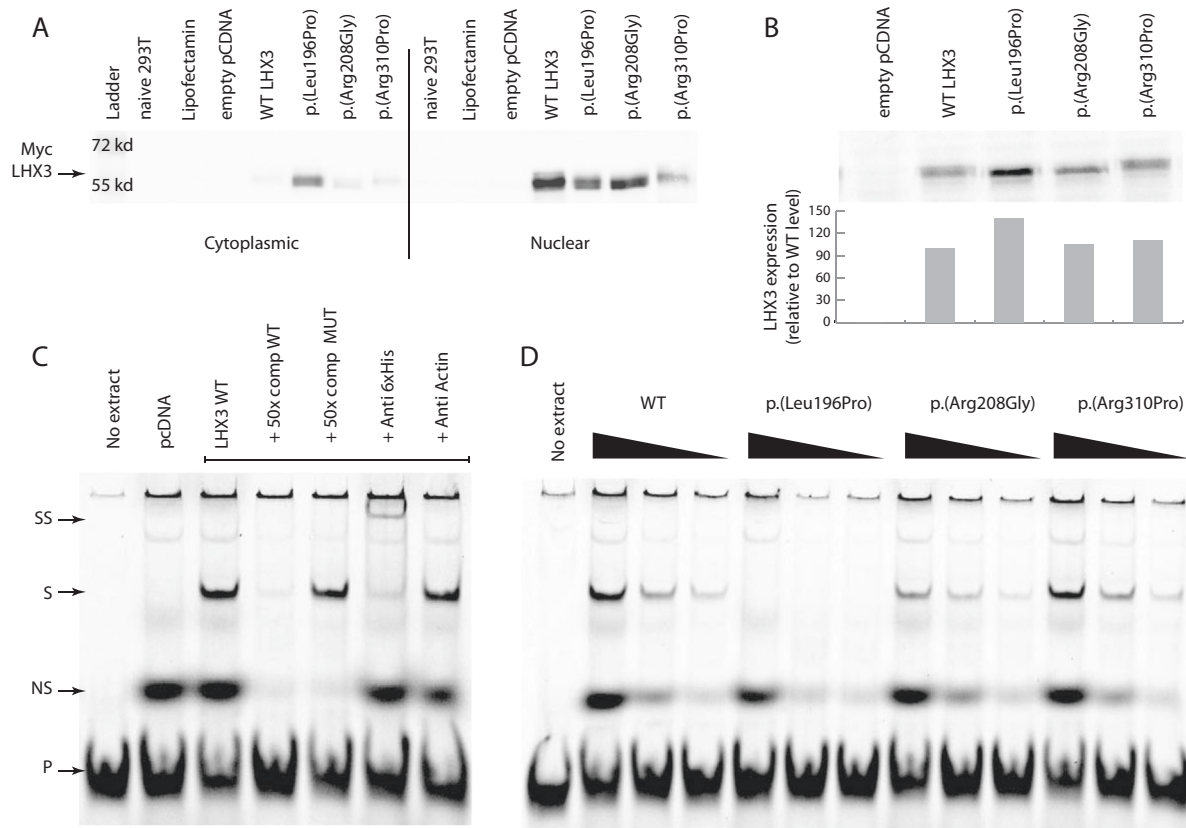


Fig. 1 **a** Western blot analysis of LHX3 proteins extracted from transfected heterologous HEK293T cells with wild type and mutated pcDNA-LHX3a-myc construct. Immunodetection of LHX3a-myc was performed using an anti-myc mouse monoclonal antibody. Left panel, cytoplasmic extracts; right panel, nuclear extracts. **b** Western blot analysis to quantify the amount of each LHX3 protein (transfected nuclear extract) for electromobility shift assay experiments (EMSA). **c** EMSA was performed with a 2xCy5 LUEGO-based probe containing the LHX3-binding site of the *alpha-GSU* promoter. Supershift (SS) was performed with a monoclonal anti-6xHis antibody. S arrow

indicates the position of the LHX3 DNA binding. P, Free probe; pcDNA, pcDNA empty vector; NS, non-specific band; 50-fold higher concentration unlabeled wild-type probe (same sequence as labeled probe) (50x comp WT), mutated probe used as a competitor (50x comp MUT). **d** EMSA was performed with decreasing (three-fold dilution series) amounts of each mutant LHX3 protein. WT LHX3: proteins from LHX3-WT-transfected HEK293T cells; p.(Leu196Pro): proteins from LHX3-p.(Leu196Pro)-transfected cells; p.(Arg208Gly): proteins from LHX3-p.(Arg208Gly)-transfected cells; p.(Arg310Pro): proteins from LHX3-p.(Arg310Pro)-transfected cells

performed with extracts from COS-1 cells (data not shown), and with an anti-LHX3 antibody (data not shown).

Electromobility shift assay

To test the binding properties of the mutants, DNA binding was assessed by EMSA analysis using a binding site of the *alpha-GSU* promoter. The same quantity of each LHX3 protein was used by varying the total amount of transfected nuclear extract (Fig. 1b). We first identified the specific band corresponding to LHX3-WT (Fig. 1c) and confirmed that the binding was specific and sensitive (as shown by competition with the WT and mutant probe) (Fig. 1c). We then compared a three-fold dilution series of each variant vs WT: LHX3-p.(Arg208Gly) and LHX3-p.(Arg310Pro) had the same binding affinity as LHX3-WT. Finally, no binding was detected for LHX3-p.(Leu196Pro) (Fig. 1d). It should be noted that the total amount of protein did not affect the

binding properties of LHX3-WT (data not shown) and that the same results were observed when the EMSA was performed with extracts from COS-1 cells (data not shown).

Transfections

The ability of the LHX3 mutants to activate the *alpha-GSU* promoter was investigated, by cotransfecting HEK293T cells with various amounts of WT and mutant LHX3 expression vectors (Fig. 2a). Both LHX3-WT and LHX3-p.(Arg310Pro) activated the *alpha-GSU* promoter in a dose-dependent manner, while LHX3-p.(Leu196Pro) and LHX3-p.(Arg208Gly) did not ($p < 0.05$ for 200 ng, $p < 0.005$ for 400 ng vs LHX3-WT). Results were comparable when the same expression vectors were tested on the *prolactin* and *beta-TSH* promoters (data not shown). As LHX3-p.(Leu196Pro) was deleterious in functional studies, and present in a heterozygous state in our patient, we

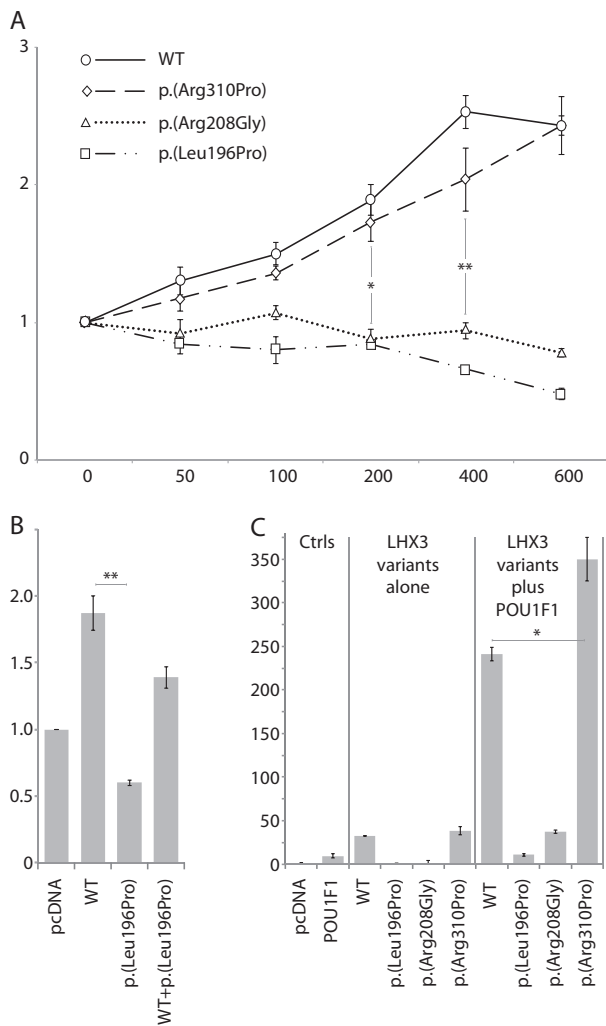


Fig. 2 Transfection studies into heterologous HEK293T cells with a luciferase reporter gene under the control of a pituitary promoter. **a** Dose response Luciferase activity of the *alpha-GSU* promoter with LHX3-WT and mutants. X-axis, dose of pGL3-*alphaGSU/Luc* plasmid (in ng). Y-axis, luciferase activity. Empty pcDNA was used as a control. **b** LHX3-WT (150 ng) and LHX3-p.(Leu196Pro) (150 ng) were cotransfected so that the total amount of DNA was comparable to two-fold LHX3-WT (300 ng). **c** Synergistic effect of POU1F1 and LHX3-WT on the prolactin promoter (hPRL-250/Luc). Cotransfections were performed with POU1F1 in combination with LHX3-WT or mutant LHX3. * $p < 0.05$; ** $p < 0.005$. Ctrl: controls; p.(Leu196Pro): LHX3-p.(Leu196Pro)-transfected cells; p.(Arg208Gly): LHX3-p.(Arg208Gly)-transfected cells; p.(Arg310Pro): LHX3-p.(Arg310Pro)-transfected cells; POU1F1:pcDNA-POU1F1, WT: LHX3-WT-transfected HEK293T cells

assessed a possible dominant-negative effect in vitro by cotransfecting LHX3-WT and LHX3-p.(Leu196Pro) along with the three promoters studied. When LHX3-WT was cotransfected with equal amounts of LHX3-p.(Leu196Pro) (150 ng each), a slight (<50%) but significant decrease in the level of activation of each promoter (compared with double the quantity of LHX3-WT, i.e. 300 ng) was observed on the *alpha-GSU* promoter ($p < 0.05$, Fig. 2b), as

well as on the *beta-TSH* and *prolactin* promoters (data not shown), suggesting haplo-insufficiency rather than a dominant-negative effect.

As LHX3 is known to synergistically interact with POU1F1 to activate the *prolactin* promoter, we tested whether this synergy was preserved with each LHX3 mutant. Synergy was observed with LHX3-p.(Arg310Pro) (350-fold activation) and was significantly increased compared to the level of activation observed when LHX3-WT and pcDNA-POU1F1 were cotransfected (240-fold activation) ($p < 0.05$). In contrast, this synergistic activity was lost with LHX3-p.(Leu196Pro) and LHX3-p.(Arg208Gly) (Fig. 2c) ($p < 0.05$ vs LHX3-WT).

Discussion

We report here the first heterozygous *LHX3* variant with likely deleterious consequences in a patient with an isolated neonatal CPHD and lack of an extra-pituitary phenotype. Symptomatic heterozygous *LHX3* variants were previously reported in one study, but as compound heterozygous variants in a child presenting with a history of severe respiratory distress, in a context of CPHD and scoliosis [21]. In this previous study, genomic analysis of the patient revealed a NM_178138.5(LHX3):c.252-3C > G p.(?) (Chr9 (GRCH37):g.139091729G > C) variant inherited from the father and a NM_178138.5(LHX3):c.353G > A (p.(Cys118Tyr) variant inherited from the mother. Interestingly, the father and the father's mother, who both had a heterozygous NM_178138.5:c.252-3C > G *LHX3* variant, presented with limited neck rotation, a clinical sign that is present in about half of the patients with *LHX3* variants, but had normal pituitary evaluation. This suggested that deleterious variants of *LHX3*, in a heterozygous state, could induce a mild phenotype. Our results support this hypothesis by describing the first case of CPHD due to a heterozygous NM_178138.5(LHX3):c.587T > C (p.(Leu196Pro)) variant. Further supporting this milder phenotype, our patient did not show any extra-pituitary anomalies, such as deafness or pituitary stalk interruption syndrome, characteristics that have been frequently reported in patients with homozygous deleterious variants of *LHX3*. Such a mild phenotype has not previously been reported in the parents of the proband carrying other *LHX3* variants, and this may be due to a low penetrance of pituitary deficiency in this context, or to a later age at the onset of pituitary deficiency (even though our proband was diagnosed immediately after birth). Such an incomplete penetrance could also be shown in the mother of our proband, who had a normal pituitary evaluation by age 40, while she was also carrying the same variant in a heterozygous state. This has already been reported by ourselves and others, for

example, in the case of another LIM domain transcription factor, LHX4 [25]. Another possibility could be that this unaffected parent may represent a mosaic. The altered function of LHX3 induced by NM_178138.5(LHX3):c.587T>C (p.(Leu196Pro)), a variant that had not been previously reported, and is in particular absent from the GnomAD database, was elucidated by our functional studies. Amino acid 196 is located in the homeodomain, a functional domain necessary for correct DNA binding; the variant appears to disrupt this domain and strongly diminish binding to DNA, as evidenced by our EMSA analysis. To reproduce the heterozygous state of the mutant in vitro, we performed transient transfections with the same quantities of LHX3-WT and LHX3-p.(Leu196Pro). As compared with double the amount of LHX3-WT, a slight (<50%) but significant decrease of promoter activation was observed in this condition, suggesting the mechanism is haplo-insufficiency rather than a dominant-negative effect. Finally, while murine models of heterozygous inactivation of *Lhx3* do not show any pituitary anomaly, it is now well accepted that murine models do not always reproduce the human disease, as has been shown for another LIM domain transcription factor, LHX4, for which heterozygous inactivation in mice does not result in a pituitary phenotype, while heterozygous variants in humans do result in a CPHD phenotype [25]. From a genetic viewpoint, we cannot definitively exclude another etiology for CPHD in this patient. However, the results of NGS confirmed the lack of presumed deleterious variants in 15 other genes known to induce CPHD. As variants in the promoter or the non-coding regions of *LHX3* have not previously been described, we sequenced only the exonic and intronic regions immediately flanking *LHX3*; furthermore, interpretation of variants in distant 5'UTR, 3'UTR, or deep intronic regions is not clear-cut. Finally, while information on copy number variation in the 16 genes, including *LHX3*, was provided by NGS analysis, excluding whole or multiexon deletion in the analyzed genes, we also performed MLPA for the samples and checked for the absence of any deletion, at least in the exons of genes present in the available MLPA kit dedicated to pituitary deficiencies (*GHI*, *LHX4*, *POU1F1*, *PROPI*, and *LHX3*).

The two other variants we report emphasize the difficulties in defining the potential affected function induced by new variants, as has been previously shown in the case of another LIM domain transcription factor, LHX4 [26]. The variant NM_178138.5(LHX3):c.929G>C (p.(Arg310Pro)) was considered to be deleterious in silico and considered as “of unknown significance” in dbSNP. However, in vitro experiments did not highlight any decreased transactivation effect of LHX3-p.(Arg310Pro) compared to LHX3-WT, despite the fact that the amino acid 310 is located in the carboxy-terminal part of the protein, a region containing the

major transactivation domain critical for pituitary gene regulation [27, 28]. LHX3-p.(Arg310Pro) was also able to interact synergistically with POU1F1 to activate the PRL promoter, in contrast to LHX3-p.(Leu196Pro). Interestingly, our functional study showed that the synergistic effect observed when LHX3-p.(Arg310Pro) was cotransfected with pcDNA-POU1F1 was superior to the one observed with LHX3-WT. As overexpression of LHX3a can lead to a developmental defect in the pituitary, and persistent expression of PROP1, a late-acting transcription factor, could lead to abnormal pituitary development [29], we cannot rule out the possibility that this increased synergistic activity could be responsible for the phenotype of the patient. We thus still suggest that LHX3-p.(Arg310Pro) should be considered as a VUS.

The third variant, NM_178138.5(LHX3):c.622C>G (p.(Arg208Gly)), has been shown by our functional studies to affect LHX3 function, in agreement with the results of the algorithms of function prediction. The phenotype was novel as it included both retinal dystrophy and corpus callosum dysgenesis. While LHX3 expression has never been demonstrated in mouse midbrain, it has been reported in a subset of bipolar cells in the retina of adult mice [30]. Our western blot data ruled out the possibility that LHX3-p.(Arg208Gly) might lead to defective nuclear localization, despite the fact that the arginine 208 residue is the first amino acid of a RRAK motif, presumed to be a nuclear localization signal [31]. However, complete mutagenesis of the *RRAK* gene, or a point variant (substitution of alanine 210 by valine), did not modify the localization of LHX3 [19]. As LHX3-p.(Arg208Gly)-binding affinity was similar to LHX3-WT, the lack of target-promoter activation could also be due to at least three alternative mechanisms: a change in the protein conformation as shown for NM_000306.3:c.537C>G (p.(Ser179Arg)) *POU1F1* variant [32]; a loss of interaction of LHX3-p.(Arg208Gly) with transcription factors such as ISL1, which are necessary for proper pituitary development [33]; or a loss of interaction of cofactors such as Sp1 or Nuclear Factor 1 which modulate LHX3 expression during development [34]. NM_178138.5(LHX3):c.622C>G (p.(Arg208Gly)) variant should thus be considered to affect the function of LHX3 in the context of pituitary deficiency as we ruled out variants of other genes currently known to be involved in CPHD by NGS. Of note, the atypical extra-pituitary phenotype presented by this patient of Iranian origin might also be due to other genetic etiologies as yet not determined.

In conclusion, this study is important for endocrinologists dealing with CPHD, as it suggests for the first time that a parent carrying a heterozygous variant of *LHX3* affecting its function, should be closely evaluated by an endocrinologist as he/she may have pituitary deficiencies that have remained undiagnosed. Such a clinical status, especially

when unrecognized, may lead to major comorbidities and could be fatal if left untreated [35]. Another important issue remains the difficulty in determining the functional activity of new variants of genes coding for transcription factors, suggesting that new tools are needed to improve the interpretation of our genetic analyses, in this era of NGS.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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