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## Heterozygous *lhx3* mutations can lead to a mild phenotype of combined pituitary hormone deficiency

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# Abstract

LHX3 is a LIM domain transcription factor involved in the early steps of pituitary ontogenesis. To date, 14 homozygous *LHX3* mutations have been reported as responsible of combined pituitary hormone deficiency in Humans. We report the first heterozygous variant of *LHX3* responsible for a milder phenotype of combined pituitary hormone deficiency. To show the pathogenicity of this L196P-LHX3 variant, heterologous HEK293T cells were transfected with plasmids encoding for wild-type or mutant LHX3. Protein expression was analysed by Western Blot, and DNA binding by Electro-mobility shift assay experiments. Target promoters of LHX3 were cotransfected with wild type or mutant LHX3 to test the transactivating abilities of the L196P variant. Our results show that *L196P-LHX3* is not able to activate target promoters in vitro as it does not bind DNA, and likely leads to a phenotype via a mechanism of haplo-insufficiency. Our study underlines the possibility that patients with a heterozygous variant of LHX3 might have pituitary deficiencies, and an extra-pituitary phenotype, possibly milder than patients with homozygous mutations of LHX3. It is thus of major importance to propose an optimal follow-up of such patients, who, up to now, were considered as safe of pituitary diseases. We finally also report the results of functional studies of 2 other variants, presumed to be deleterious in silico: one in a homozygous state (R208G-LHX3), for which we observed a decreased transactivating ability in vitro, concordant with in silico analysis; the other (R310P-LHX3), in a heterozygous state, and for which we did not manage to identify any deleterious effect, thus presumed to be non-pathogenic. Our study thus also emphasizes the issues in clearly defining the pathogenicity of new variants, for which functional studies still seem necessary.

# Introduction

Combined pituitary hormone deficiency (CPHD) is defined as the presence of constitutional anterior pituitary hormone deficiency with or without syndromic features. Mutations of genes involved in late pituitary development (such as *PROP1*, *POU1F1* or *TBX19*) are responsible for endocrine phenotypes without extra-pituitary defects (1). Mutations of early expressed genes, such as *LHX3*, *LHX4* or *HESX1*, are commonly associated with extra-pituitary malformations such as neck rotation abnormalities, hearing loss or septo-optic dysplasia.

In mice, the proper expression of the LIM (LIN-11, Isl1 and MEC-3)-homeodomain transcription factor *lhx3* is necessary for pituitary development and function (2) (6,7). *Lhx3* is necessary for mice survival: mice with a homozygous inactivation of *lhx3* (*lhx3*<sup>-/-</sup>) die immediately after birth; their pituitaries are aplastic and lack the posterior and intermediate lobes (2, 10). In contrast, *Lhx3*<sup>+/-</sup> mice have normal pituitary and no specific phenotype (11). The overexpression of 2 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*, *beta-TSH*, *beta-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 *beta-TSH* and *prolactin* (9). All these evidence are in favor of a major role of *lhx3* during pituitary development. Of note is that *lhx3* is also involved in the development of the motor neurons (3) and the inner ear (4), which explains the complex phenotype of some patients carrying *LHX3* mutations.

In humans, homozygous mutations of *LHX3* lead to CPHD. To date 14 autosomic recessive mutations of *LHX3* have been reported in patients with CPHD (12-21). All the mutations were identified in exons, excepted one located in an intronic region (17). Patients bearing *LHX3* mutations displayed constant somatotroph, thyrotroph and gonadotroph, and inconstant corticotroph deficits. On MRI, pituitary was described as hypoplastic or aplastic in 60%, normal in 10% and hyperplastic in 30% of cases. The pituitary phenotype was frequently associated with rigid neck (70% of patients), vertebra anomalies and more or less severe hearing loss (50% of cases).

In this study, we report the first heterozygous mutation of *LHX3* responsible for CPHD. As a comparison, we also report the results of the functional studies of 2 newly identified homozygous variants of *LHX3* that were considered as deleterious by *in silico* analysis.

# Patients 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, a genetic screening of *LHX3* mutations was performed specifically in patients with at least GH, TSH and LH/FSH deficits. Patients with a known postnatal cause of acquired hypopituitarism were excluded. Hormonal studies and intracranial imaging were performed in all patients in each referring medical center. On MRI, malformations were systematically sought and recorded. After written informed consent was given, blood samples were collected from patients and, whenever possible, first-degree relatives. Informed written consent was obtained from the parents, caretakers or guardians on behalf of the minor/children enrolled in the study. The study was approved by the Ethics committee of the University of Aix-Marseille II (France).

## Screening for *LHX3* mutations

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 on request). Amplification was carried out using the Hot Start Taq polymerase kit protocol (Quiagen GmbH, Hilden, Germany). Sequencing was performed with a 3130 XL Genetic analyzer (LifeTechnologies, New-York, USA). Sequences were analyzed with the Variant Reporter software (LifeTechnologies, New-York, USA).

## *In silico* analysis

Alignment of amino acids sequences was performed with UniProtKB ([www.uniprot.org](http://www.uniprot.org)). A prediction software from Aix-Marseille University ([www.umd-hts.eu/WHTS9](http://www.umd-hts.eu/WHTS9)) was used to determine the potential pathogenicity of each allelic variant as previously detailed.

## Plasmids constructs and mutagenesis

The 550 bp alphaGSU promoter (pGL3-alphaGSU/Luc) and the 1273 bp betaTSH 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 a myc tag in its C-terminal part and inserted into the CMV-driven eukaryotic expression vector pcDNA3 (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 pcDNA3 expression vector. The *L196P*, *R208G* and *R310P* mutations in the pcDNA3-*LHX3a*-myc construct were generated by PCR, with the QuickChange Mutagenesis Kit (Stratagene, La Jolla, CA, USA) and the following commercially synthesized oligonucleotides (IDT San Jose, California, USA); mutations are indicated in the sense strand (in bold):

- L196P: 5'- CTCTCGTCCGAGACGGGCC**CGG**ACATGCGCGTGGTGC-3';
- R208G: 5'- GCAGGTTTGGTTCCAGAACGGCC**GG**CCAAGGAGAAGAGG-3';
- R310P: 5'-GAGCAGTACCGAGAGCTGC**CT**CCCGGCAGCCCCTACGG-3'.

## **Cell culture and transfections**

HEK293T cells were grown in 12-well plates in DMEM supplemented with 10% fetal bovine serum. Cells were transfected in serum-free medium with the Lipofectamine reagent (Invitrogen) according to the manufacturer's instructions. HEK293T cells were prepared and assayed for luciferase activity 48h after transfection, using the Dual-luciferase Assay system (Promega). Each transfection experiment, in triplicate, was performed at least three times.

## **Nuclear cell extracts**

Nuclear proteins were extracted from the HEK293T cells 48h following transfection in 100 mm dishes as previously described (23). Protein contents in the lysates were measured by colorimetric analysis using Lowry protein assay (Bio-Rad Laboratories Inc.).

## **Western Blot**

Nuclear protein extracts were resolved on a 9% SDS-PAGE gel. After transfer on a PDVF membrane (Perkin Elmer, Waltham, MA, USA), immunodetection of LHX3a-myc was performed using an anti-myc mouse monoclonal antibody (1/1000; provided by Dr JV Barnier, NCBM, Marseille, France) and a fluorescent anti-mouse Qdot antibody (ThermoFisher scientific). Membranes were analyzed with a G:BOX-iChemi (Syngene).

## **Electrophoretic Mobility Shift Assay**

EMSA were carried out with wild-type (wt) and mutant LHX3 and the <sup>32</sup>P-labeled double-stranded oligonucleotide 5'-ACATTAGGTACTTAGCTAATTAAATGTG-3', containing the LIM-specific binding site of the alphaGSU promoter according to the protocol previously described (23). The gel was then placed against X-ray film for autoradiography.

## **Statistical analysis**

Data points were compared using a one-tailed Student's t-test for paired samples using XLStat 2013.4.05 (Paris, France). Values were considered significantly different when  $p < 0.05$ .

# RESULTS

## Individual data of the patients bearing *LHX3* mutations

### **Pedigree A: *L196P* *LHX3* allelic variant (heterozygous)**

The propositus is an Italian male with a neonatal panhypopituitarism and a micropenis. Cerebral MRI was normal. The mother of the propositus was carrying the same variant and she had normal pituitary evaluation by age 40.

### **Pedigree B: *R208G* *LHX3* allelic variant (homozygous)**

The propositus is an Iranian girl presenting with a neonatal panhypopituitarism. The phenotype also includes a retinal dystrophy and low-set ears. Cerebral MRI showed a cystic anterior pituitary and a corpus callosum digenesis. Her parents were consanguineous. No genetic analysis was available for any other family member.

### **Pedigree C: *R310P* *LHX3* allelic variant (heterozygous)**

The propositus is a boy from Argentina with a neonatal panhypopituitarism associated with microcephaly and a micropenis. Cerebral MRI showed anterior pituitary hypoplasia, an ectopic posterior pituitary and an interrupted pituitary stalk. No genetic analysis was available for any other family member.

## ***In silico* analyses**

All of the modified *LHX3* amino acid residues are conserved in known *LHX3* sequences in mammals, mice, chicken and fish. Prediction softwares suggested that the 3 allelic variants were presumed to have a damaging effect for the protein.

## ***In vitro* analyses**

### **Western Blot**

We evaluated the production of the wt and mutant *LHX3* proteins by western blot using nuclear extracts from transfected HEK293T cells (Fig. 1A). We detected bands of the expected size for *LHX3*-wt-myc, *LHX3*-*L196P*-myc, *LHX3*-*R208G*-myc and *LHX3*-*R310P*-myc.

### **Electro mobility shift assay**

To test the binding properties of the mutants, DNA binding was assessed by EMSA analysis using a radiolabeled *LHX3*-binding site of the alpha-GSU promoter (Fig. 1B). Whereas *LHX3*-wt, *LHX3*-*R208G* and *LHX3*-*R310P* bound the probe, no binding was detected for *LHX3*-*L196P*.

### **Transfections**

We investigated the ability of the *LHX3* mutants to activate the alpha-GSU promoter, by cotransfecting HEK293T cells with various amounts of wt and mutant *LHX3* expression vectors (Fig. 2A). Both *LHX3*-wt and *LHX3*-*R310P* activated the alpha-GSU in a dose-dependent manner, while *LHX3*-*L196P* and *LHX3*-*R208G* did not ( $p < 0.001$  vs *LHX3* wt). Results were comparable with the PRL and beta-TSH promoters (data not shown). As *LHX3* is known to interact synergistically with Pou1f1 to activate the PRL promoter, we tested whether the synergy was kept with each *LHX3* mutant. This synergy was observed with *LHX3* *R310P* (fold activation 350) and was significantly increased compared to the level of activation observed when *LHX3* wt and Pou1f1 are cotransfected (fold activation

240) ( $p < 0.05$ ). In contrast, the synergistic activity was lost with LHX3 L196P and LHX3 R208G (Fig. 2B) ( $p < 0.05$  vs LHX3 wt).

As two of the mutations, LHX3-R310P and LHX3-L196P were in a heterozygous state, we assessed this condition *in vitro* by cotransfecting LHX3-wt and both mutants along with the three promoters studied (Fig. 2C). When LHX3-wt was cotransfected with equal amount of LHX3-L310P, the level of activation of the alpha-GSU promoter was the same as when 2x LHX3 wt were transfected. When LHX3-wt was cotransfected with equal amount of LHX3-L196P, a slight but significant decrease of the level of the activation of each promoter was observed on the alpha-GSU, beta-TSH and prolactin promoters ( $p < 0.05$ ).



## Discussion and conclusion

We report here the first heterozygous *LHX3* mutation with deleterious consequences in a patient with a neonatal CPHD, but the lack of an extra-pituitary phenotype. Symptomatic heterozygous *LHX3* mutations were previously reported, but as compound heterozygous mutations in a child presenting with a severe history of respiratory distress, in a context of CPHD and scoliosis (20). Analysis of the patient revealed a *c.252-3 C>G* variant inherited from the father and a *c.353 G>A* variant inherited from the mother. Interestingly, the father and the father's mother who both had a heterozygous *c.252-3 C>G LHX3* mutation presented with a limited neck rotation, a clinical sign that is present in about half of the patients with *LHX3* mutations, but had a normal pituitary evaluation. This suggested that deleterious variants of *LHX3* in a heterozygous state could induce a mild phenotype. Our results thus confirm this hypothesis by describing the first case of CPHD due to a heterozygous *LHX3* mutation. Interestingly however, our patient did not have any extra-pituitary anomalies, such as deafness, abnormal neck rotation or pituitary stalk interruption syndrome, characteristics that had been frequently reported in patients with homozygous deleterious variants of *LHX3*. Such a mild phenotype has never been reported before in the parents of the propositus carrying other *LHX3* mutations, and this could be due to a low penetrance of pituitary deficiency in this context, or to a later age at pituitary deficiency appearance (even though our propositus was diagnosed immediately after birth). The pathogenicity of *LHX3 L196P*, a variant that had never been reported before in the EXAC database (data not shown), is however ascertained by our functional studies. The amino-acid 196 is located in the homeodomain, a functional domain necessary for proper DNA binding, which explains why *LHX3 L196P* was unable to bind the consensus binding site of the alpha-GSU promoter. To reproduce the heterozygous state of the mutant *in vitro*, we performed transient transfections with the same amounts of *LHX3* wt and *LHX3 L196P*. A decrease of the promoter activation was observed in this condition (compared with a double amount of *LHX3* wt), though inferior to 50%, suggesting an haplo-insufficiency mechanism rather than a dominant negative effect. Of note is that a competitive effect between *LHX3* wt and *LHX3 L196P* for the promoter binding site is excluded, because *LHX3 L196P* is not able to bind DNA.

The 2 other variants we report emphasize the difficulties in defining the pathogenicity of new variants, as previously shown for another LIM domain transcription factor, *LHX4*. Our results indeed suggest that *LHX3 R310P* is likely a non deleterious variant, in contrast with the results of algorithms predicting the pathogenicity. The *LHX3 R310P* heterozygous variant is located in the carboxy-terminal part of the protein. This region of *LHX3* contains the major transactivation domain critical for pituitary gene regulation (24, 25). However, several evidence are in favor of a non deleterious variant. *In vitro* experiments did not highlight any decreased transactivation effect of *LHX3 R310P* compared to *LHX3* wt. *LHX3 R310P* is also able to interact synergistically with Pit-1 to activate the PRL promoter. Finally, 2 other variants have been reported for the amino acid 310, and they were not considered as deleterious (EXAC database, data not shown). Interestingly, our functional study showed that the synergistic effect observed when *LHX3 R310P* was cotransfected with *Pou1f1* was superior to the one observed with *LHX3* wt. As overexpression of *lhx3a* can lead to pituitary development defect, and overexpression of *PROP1*, a late-acting transcription factor, could lead to abnormal pituitary development, we can not be sure that this increased efficacy could not be responsible for the phenotype of the patient. In contrast, the *LHX3 R208G* mutation has

been shown as deleterious by our functional studies, concordant with the results of the algorithms of pathogenicity prediction. LHX3 R208G might lead to a defective nuclear localization. The 208 arginine residue is the first amino acid of a RRAK motif, presumed to be a nuclear localization signal (27). However, a complete mutagenesis of the RRAK, or a point mutation (alanine 210 by valine) did not modify the localization of LHX3 (18). Our western blot results performed with nuclear extracts also argue against this hypothesis. As we did not show any alteration of the DNA binding properties using EMSA, the lack of target-promoter activation might be due to 3 mechanisms: a change in the protein conformation as shown for S179R POU1F1 variant; a loss of interaction of LHX3 R208G with transcription factors such as ISL1, necessary for proper pituitary development (28); a loss of interaction of cofactors such as Sp1 or Nuclear Factor 1 which modulate LHX3 expression during development (29).

To conclude, this study is of importance for endocrinologists dealing with CPHD, as it suggests for the first time that a parent carrying a heterozygous *LHX3* variant should be closely evaluated by an endocrinologist as he/she might have pituitary deficiencies, a clinical status that leads to major comorbidities and can be fatal if left untreated. Another important issue remains the difficulty in determining the pathogenicity of new variants of genes coding for transcription factors, suggesting that new tools are needed to improve our genetic analyses.

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## Figures legend

**Figure 1:** A. Western Blot. Western blot analysis of wild-type and mutant LHX3 proteins from transfected heterologous human embryonic kidney 293T cells. Immunodetection of LHX3a-myc was performed using an anti-myc mouse monoclonal antibody. B. Electromobility Shift Assay Experiments. EMSA was performed with a radiolabeled LHX3-binding site of the alpha-GSU promoter. Supershift was performed with a monoclonal anti-myc antibody. FP, free probe. White arrow indicates the position of the LHX3 DNA binding. Black arrow indicates the supershift.

**Figure 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 plasmid in ng. Y axis, luciferase activity. Plain line with circles, WT LHX3; dotted line with losanges, R310P LHX3; Pointed line with triangles: L196P LHX3; Dotted and pointed line with squares: R208G. Empty PcDNA was used as a control B. Cotransfection of WT LHX3 and L196P mutant did not show any dominant negative effect on prolactin promoter. LHX3 and L196P were cotransfected so that the whole amount of DNA was comparable to 2 fold WT LHX3. C. Synergistic effect of PIT-1 and WT LHX3 on the prolactin promoter. Cotransfections performed with PIT-1 and WT or mutant LHX3 did not show any synergy for L196P and R208G.

## Tables

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

Mutation	L196P	R208G	R310P
Year of birth	2005	2007	2003
Country	Italy	Iran	Argentina
Medical history of hypopituitarism in family	No	No	No
Inheritance	Heterozygous	Homozygous	Heterozygous
ACTH deficiency	Yes (neonatal)	Yes (neonatal)	No
TSH deficiency	Yes (neonatal)	Yes (neonatal)	Yes (3 yrs)
FSH/LH deficiency	NE	NE	NE
GH deficiency	Yes (neonatal)	Yes (neonatal)	Yes (2 yrs)
Anterior pituitary on MRI	Normal	Hypoplastic with a cyst	Normal
Posterior pituitary MRI	Normal	Normal	Ectopic
Pituitary stalk MRI	Normal	Normal	Thin
Associated malformations	No	Low-set ears, retinal dystrophy, corpus callosum digenesis	Cleft lip and palate

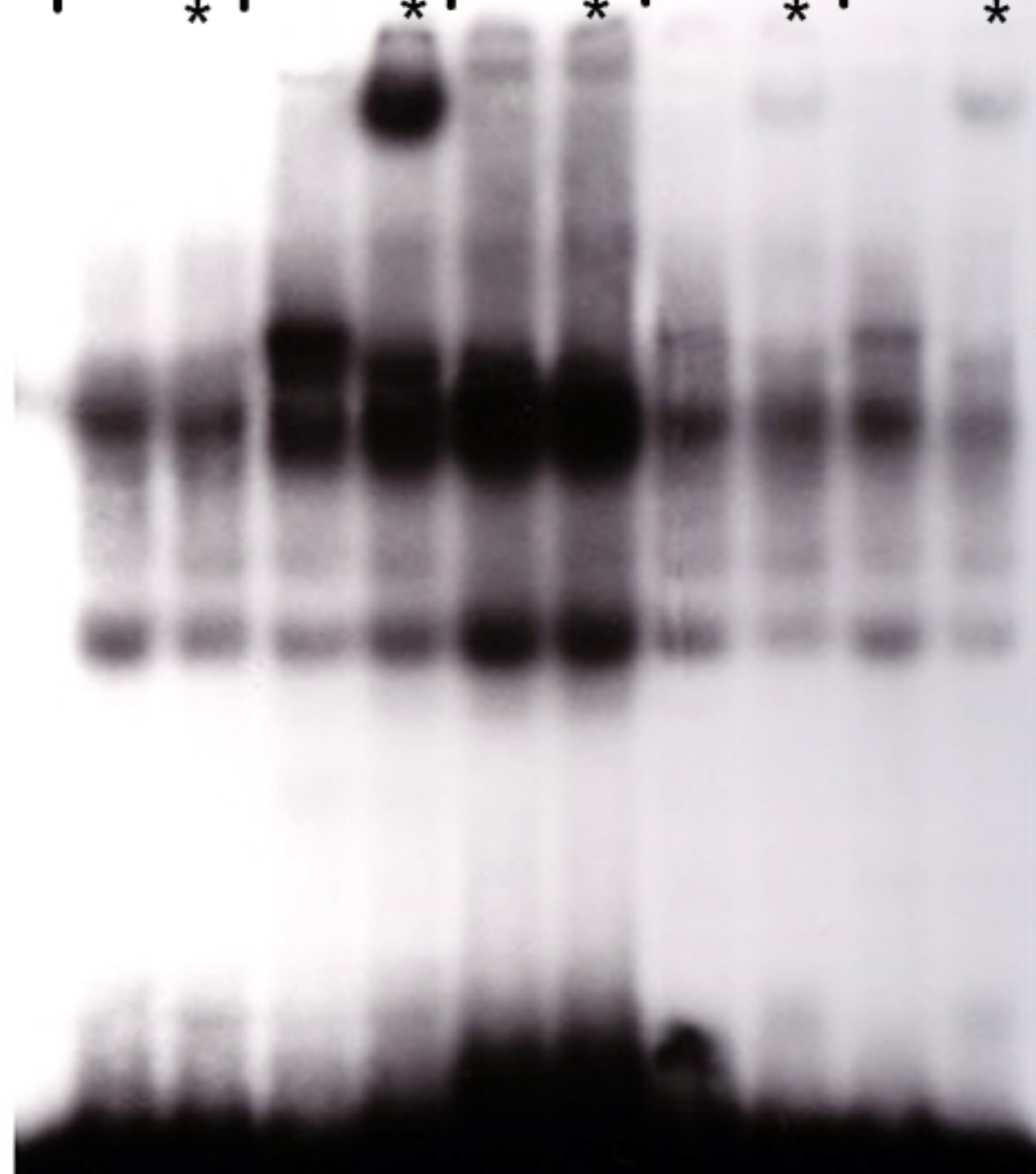
For pituitary deficiencies, data between brackets represent age at diagnosis. Complete GH deficiency was defined as GH response after stimulation below 10 mUI/liter. Corticotroph deficiency was defined as plasma cortisol value below 500 nmol/liter after insulin test stimulation. Gonadotroph axis was investigated only in patients of postpubertal age, *ie.* older than 15 years for female and 17 years in male subjects (NE, not evaluated). FSH-LH deficiency was diagnosed on the basis of delayed or absent pubertal development with low serum testosterone or estradiol levels and blunted LH/FSH response to a GnRH stimulation test. Thyrotroph deficiency was defined as low or normal basal TSH levels associated with low T4 levels.

Non transfected cells      PcDNA      LHX3 WT      L196P      R208G      R310P



A

LHX3  
FP   PcDNA   WT   L196P   R208G   R310P  
\*   \*   \*   \*   \*



B



