CASE REPORT

A novel *OTX2* mutation in a patient with combined pituitary hormone deficiency, pituitary malformation, and an underdeveloped left optic nerve

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Abstract

Orthodenticle homolog 2 (*OTX2*) is a homeobox family transcription factor required for brain and eye formation. Various genetic alterations in *OTX2* have been described, mostly in patients with severe ocular malformations. In order to expand the knowledge of the spectrum of *OTX2* mutation, we performed *OTX2* mutation screening in 92 patients with combined pituitary hormone deficiency (CPHD). We directly sequenced the coding regions and exon–intron boundaries of *OTX2* in 92 CPHD patients from the Dutch HYPOPIT study in whom mutations in the classical CPHD genes *PROP1*, *POU1F1*, *HESX1*, *LHX3*, and *LHX4* had been ruled out. Among 92 CPHD patients, we identified a novel heterozygous missense mutation c.401C>G (p.Pro134Arg) in a patient with CPHD, pituitary malformation, and an underdeveloped left optic nerve. Binding of both the wild-type and mutant *OTX2* proteins to bicoid binding sites was equivalent; however, the mutant *OTX2* exhibited decreased transactivation. We describe a novel missense heterozygous *OTX2* mutation that acts as a dominant negative inhibitor of target gene expression in a patient with CPHD, pituitary malformation, and optic nerve hypoplasia. We provide an overview of all *OTX2* mutations described till date, which show that *OTX2* is a promising candidate gene for genetic screening of patients with CPHD or isolated GH deficiency (IGHD). As the majority of the *OTX2* mutations found in patients with CPHD, IGHD, or short stature have been found in exon 5, we recommend starting mutational screening in those patients in exon 5 of the gene.

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Introduction

Orthodenticle *Drosophila* homolog 2 (*OTX2*; MIM 600037), is a homeobox family transcription factor, which is required for brain and eye formation. *OTX2* is located in chromosome 14q and has five exons of which three are coding. There are two known isoforms: a (NM_021728.2; NP_068374.1) and b (NM_172337.1; NP_758840.1); isoform b is the major product of the gene and has eight fewer amino acids than isoform a. In the mouse, during early development, *Otx2* is expressed in the forebrain and midbrain and has a role in the development of the brain, face, and skull (1, 2, 3).

Various genetic alterations in *OTX2* have been described, including interstitial deletions (4, 5), microdeletions (6, 7), frameshift, and point mutations (8). The majority of the alterations are found in patients with severe ocular malformations such as anophthalmia (AO),

microphthalmia (MO), Leber congenital amaurosis, or coloboma (6, 8, 9, 10). *OTX2* has been shown to be important for the regulation of *HESX* homeobox 1 (*HESX1*) (11, 12), one of the transcription factors involved in pituitary development. As *HESX1* mutations have been described in patients with isolated GH deficiency (IGHD) and combined pituitary hormone deficiency (CPHD), several recent studies also performed mutation and deletion screening of *OTX2* in patients with variable degrees of pituitary dysfunction (7, 13, 14, 15, 16, 17). In the majority of the patients, but not all (14), eye malformations were also present.

CPHD is any combination of two or more anterior pituitary hormone deficiencies. Mutations in several genes encoding the transcription factors involved in pituitary cell differentiation have been associated with CPHD (for review, see (18)). However, in the majority of CPHD patients, causative gene mutations remain

unknown. Thus, in order to expand the mutation spectrum of CPHD, we performed mutation analysis of *OTX2* in a cohort of 92 CPHD patients from the Dutch HYPOPIT study. We describe a new *OTX2* mutation found in one of our patients with CPHD, who also had pituitary malformation and an underdeveloped left optic nerve. In addition to this new finding, we provide an overview of all phenotypic and genetic data reported to date.

Materials and methods

DNA samples were collected from 92 patients with CPHD, who participated in the Dutch HYPOPIT study (19). These patients had been recruited from the Endocrinology Departments of six university and two non-university Hospitals and had been registered in the Dutch National Registry of Growth Hormone Treatment between 1992 and 2003. All patients had deficiencies of GH and of one or more additional hormonal axes. Deficiencies of hypothalamic–pituitary–thyroidal, -adrenal, and -gonadal axes were defined by an abnormal TRH test or TSH levels that were low or inadequately low free-tetraiodothyronine (FT₄); abnormal CRF/ACTH/glucagon test or ACTH levels that were low or inadequately low cortisol and LH, FSH, estrogen/testosterone, or inappropriately low gonadotropin response to LHRH for age or lack of spontaneous puberty after the age of 14 years. Prolactin deficiency was defined as abnormal prolactin during random sampling or TRH testing. Reference values from the individual hospitals were used. Exclusion criteria included patients with a known cause of CPHD, such as a brain tumor, brain surgery, brain radiation, or known syndromes. Written informed consent was obtained from all participating patients and their parents or legal guardian.

DNA was isolated from the whole blood collected in EDTA tubes according to standard procedures. *OTX2* (NM_021728.2) PCR amplification and sequencing was performed as described previously (20).

Generation of plasmids

The *OTX2* cDNA was cloned into the expression vector pSG5, and the multiple bicoid binding site was placed into the luciferase-containing pGL2 promoter vector (Promega Corp.) (14). The *OTX2* Pro134Arg mutation was designed according to the QuickChange Site-direct mutagenesis protocol (Stratagene) after introducing a single base change using specific primers (available on request). After DNA amplification and Maxiprep kit purification (Qiagen), the sequence, orientation, and presence of the mutation in the plasmid were confirmed by DNA sequencing.

Electrophoretic mobility shift assay

Electrophoretic mobility shift assay (EMSA) was performed with *OTX2* recombinant proteins and ³²P-labeled DNA fragments as described previously (14). Wild-type (WT) and Pro134Arg mutation proteins were synthesized using the TNT-coupled transcription and translation reticulocyte lysate system with T7 polymerase, according to the manufacturer's protocol (Promega Corp.). To determine *OTX2* DNA binding, a consensus *OTX2* DNA binding site was used. These oligonucleotides were γ³²P-end labeled with T₄ polynucleotide kinase. For each EMSA, *in vitro*-translated proteins were mixed with radiolabeled probe for 30 min at room temperature, along with deoxyinosine–deoxycytosine, salmon sperm, and binding buffer (50 mM KCl, 20% glycerol, and 20 mM HEPES (pH 7.6–7.8)). Each sample was then separated by gel electrophoresis on a 5% nondenaturing acrylamide gel and analyzed by autoradiography. The *OTX2* complexes were supershifted with a polyclonal *OTX2* antibody. The binding was competed by addition of the unlabeled oligonucleotide in excess. ³⁵S-Methionine was added to our reticulocyte lysate system to confirm the efficiency of protein synthesis. The radiolabeled proteins were resolved on a 10% denaturing acrylamide gel and analyzed by autoradiography.

Transient transfection and cell culture

Transient transfections were performed in 293T and GN cell lines. Cells were maintained in DMEM high glucose 1 × (4.5 g/l D-glucose; Life Technologies, Inc.), supplemented with L-glutamine, 1% antibiotic–antimycotic (100×; Life Technologies), 110 mg/l sodium pyruvate, and 10% fetal bovine serum (Life Technologies). Cells were grown at 37 °C in 5% CO₂ and were transfected at 40–60% confluency. Total DNA was kept constant, and nonspecific effects of viral promoters were controlled using the empty pSG5 vector (pSG5EV). Luciferase activity in relative light units was measured at 48 h using the Lumat LB 9507 (Berthold Technologies, Oak Ridge, TN, USA).

293T transient transfections were performed in six-well tissue culture plates using lipofectamine reagent (Invitrogen). For functional studies, the pSG5-m*OTX2* cDNA or the pSG5-MUT m*OTX2* cDNA was used. A total of 125 ng of a multiple bicoid binding site promoter luciferase vector (or the pGL2 empty vector (pGL2EV)) was cotransfected with 125 ng WT *OTX2*, 62.5 ng pSG5EV/62.5 ng WT *OTX2*, 31.25 ng pSG5EV/62.5 ng WT *OTX2*/31.25 ng MUT *OTX2*, 62.5 ng WT *OTX2*/62.5 ng MUT *OTX2*, or 125 ng MUT *OTX2*. This same experiment design was repeated using GN cells.

Transfections were performed in triplicate for each condition within a single experiment, and experiments were repeated at least three times using different

plasmid preparations for each construct. The relative luciferase activity for each control (pGL2EV) was set to 1, and results were expressed as fold-promoter activation and represented as the s.e.m. of representative experiments.

Statistical analysis

Transient transfection results are expressed as the s.e.m. Statistical analysis was performed using GraphPad Prism 4 (GraphPad Software, Inc., San Diego, CA, USA). The data were normalized to empty vector expression and graphs depict fold change over empty vector. Group means were compared using single ANOVA and Tukey's multiple comparison test, with $P < 0.05$ considered statistically significant.

Results

We directly sequenced the complete coding region and intron–exon boundaries of *OTX2* in DNA samples from 92 patients with CPHD, in whom mutations in the classical CPHD genes *PROP1*, *POU1F1*, *HESX1*, *LHX3*, and *LHX4* had been ruled out (19). Clinical characteristics and magnetic resonance imaging (MRI) data of the patients have been published previously (19).

In one patient, we identified a new missense mutation c.401C>G in exon 5, which replaces proline (CCC) with arginine (CGC) at amino acid position 134 (Fig. 1A).

Figure 1 *OTX2* mutation analysis in patients with CPHD. Pedigrees of the patients with missense and silent mutations (the black arrow indicates index case) and representative chromatograms of sequences.

The mutation is heterozygous and inherited from the phenotypically normal father. Additionally, we identified a synonymous variant c.792C>T ($p.=$) in another CPHD patient (Fig. 1B). Finally, our sequencing results showed two known polymorphisms: rs2277499 in exon 4 (minor allele frequency 36%) and rs171978 in exon 5 (minor allele frequency 7%).

Clinical features

The patient carrying the *OTX2* p.Pro134Arg mutation is a male born from Dutch Caucasian non-consanguineous parents after 40 weeks of gestation. His birth length was 50 cm and his birth weight 3600 g. He had neonatal hypoglycemia. His MRI showed a normal anterior pituitary, but an ectopic posterior pituitary, an absence of pituitary stalk, and an underdeveloped left optic nerve. At the age of 8 years, clonidine test showed a GH peak of 3.4 mU/l (normal peak above 20 mU/l). Before the initiation of GH treatment, his insulin-like growth factor 1 (IGF1) was 1.9 nmol/l (-5.3 SDS) and IGFBP3 0.6 mg/l (-6.7 SDS). GH supplementation was started at the age of 8 years when his height was 1.15 m (-3.2 SDS). At the initiation of GH treatment, his bone age delay was 3.5 years. Central hypothyroidism was detected at the age of 9 years, based on a FT₄ level of 8.0 pmol/l (normal range 12–26 pmol/l) and an abnormally low TSH for this low FT₄ (2.7 mU/l); thyroid hormone supplementation was then started. Before the initiation of GH treatment, morning cortisol levels were normal (at 8 h, 0.22 μ mol/l), but at the age of 11 years, the analysis was repeated and he had low cortisol levels: at 8 h, 0.04 μ mol/l (reference 0.2–0.8 μ mol/l) and 14 h, 0.06 μ mol/l (reference 0.1–0.4 μ mol/l), ACTH was also low (12 ng/l, reference 20–80 ng/l), and therefore hydrocortisone treatment was started. At the age of 13 years, he was still totally prepubertal (Tanner stage G1 P1) with low LH (1.0 U/l) and FSH (1.8 U/l) values; at the age of 13 years and 10 months, he was still prepubertal, with a low testosterone for his age (<0.1 nmol/l); induction of puberty was started. The patient has complete pituitary hormone deficiency and continued GH treatment after reaching adulthood. He has severe behavioral problems. The father's adult height SDS is -1.0 (174 cm), and the mother's adult height SDS 0.8 (174 cm).

Functional studies

We performed structural and functional studies to determine the mechanism by which the *OTX2* Pro134Arg mutation affects target gene expression. EMSA showed that both mutant and WT proteins bind to the *OTX2* consensus site (Fig. 2A); the amount of protein synthesized was equivalent between WT and the mutant (data not shown).

The ability of WT and mutant Pro134Arg *OTX2* protein to activate gene expression was measured using

luciferase reporter gene assay systems. The construct containing the multiple bicoid binding site fused to a luciferase reporter gene was cotransfected with expression plasmids containing either WT or mutant OTX2 into a heterologous 293T human embryonal cell line (Fig. 2B) or GN cell line, a GNRH expressing

neuronal cell line (Fig. 2C). In 293T cells, WT OTX2 induced a 1.9 ± 0.08 -fold increase (48%) in luciferase activity relative to expression from pSG5EV. Transfection of equivalent amounts of WT and empty expression vector similarly demonstrated a 1.5-fold increase (34%) in transactivation. While maintaining a constant amount of WT *Otx2*, increasing amounts of mutant OTX2 plasmid (ratios of 1:2 and 1:1, WT:mutant DNA) were cotransfected, resulting in significant, (40 and 53% respectively) inhibition of luciferase expression. Further inhibition (65%) was seen with transfection of the mutant OTX2 plasmid alone. Cotransfection of WT OTX2 and the multiple bicoid binding site reporter luciferase construct into GN cells induced a 2.25 ± 0.17 increase (56%) in transactivation. Similar to the studies on 293T cells, transfecting a constant amount of WT OTX2 with increasing amounts of mutant OTX2 at ratios of 1:2 and 1:1 led to a decrease in luciferase expression (77.4 and 82.3% inhibition respectively). Cotransfection of equal amounts of WT and mutant OTX2 demonstrated an 83% decrease in expression.

Discussion

We performed OTX2 mutation screening by direct sequencing in 92 Dutch CPHD patients. We identified a novel missense mutation, p.Pro134Arg, in a patient with pituitary malformation, complete pituitary hormone deficiency, an underdeveloped left optic nerve, and severe behavioral problems. The patient's father is a carrier of the same mutation. He has a completely unremarkable phenotype and there are no other affected members in the family. The same amino acid was mutated in a patient published by Ragge *et al.* (8), but in that patient, the change was to alanine. The fact that the unaffected mother of Ragge's patient had the same mutation and that Chatelain *et al.* (21) could not demonstrate the negative effect of the mutant *in vitro* suggests that the p.Pro134Ala sequence variant probably was not a major contributor to the phenotype. Importantly, alanine is a neutral, nonpolar, and

Figure 2 Mutant Pro134Arg *Otx2* does not affect protein DNA binding but acts as dominant negative to inhibit target gene expression. (A) EMSA was performed using the consensus OTX2 binding sequence incubated with *in vitro*-transcribed and -translated empty vector (EV), wild-type OTX2 (WT), or OTX2 Pro134Arg mutant (MUT) proteins. Transient transfection studies were performed in a heterologous cell line, 293T (B), or a GNRH expressing cell line, GN (C). The OTX2 multiple bicoid binding site luciferase reporter construct (pGL2) was cotransfected along with expression vectors containing WT OTX2, MUT OTX2, or empty pSG5. Transfection with the WT OTX2 expression vector increased relative luciferase expression compared with pGL2 in both cell lines. In addition, in both cells lines, WT OTX2 concentration was held constant as increasing amounts of MUT OTX2 expression vector were cotransfected. Each independent experiment was performed in triplicate. The graphs show the mean $\pm$ S.E.M. of the fold change from at least five representative experiments. For each experiment, the coefficient of variation values was $<10\%$, $*P < 0.001$.

hydrophobic amino acid whereas arginine is basic, positively charged, and hydrophilic. The difference in chemical characteristics between these amino acids suggests that the p.Pro134Arg mutation may have a different functional impact than p.Pro134Ala.

Furthermore, we identified a new silent mutation, c.768C>T, not described till date in any control or patient population. None of the newly identified variants were present in the latest update (September 2011) of 1000 Genomes Project (<http://browser.1000genomes.org>), where 1092 individuals were sequenced with low coverage and exome sequencing. Besides, several research groups previously sequenced OTX2 in large European control populations: 164 controls were sequenced by Ragge *et al.* (8), 96 controls by Wyatt *et al.* (6), and 181 controls were sequenced by Henderson *et al.* (15). The two variants we describe in the present work were never reported in any of these studies.

Our CPHD cohort included three CPHD patients with eye malformations. Apart from the CPHD cohort, the HYPOPIT study includes an IGHD cohort of which two patients had eye malformations. We directly sequenced all 92 CPHD patients as well as the two IGHD patients with eye malformations and found that both were WT for OTX2. Table 1 shows the clinical characteristics of all five, CPHD and IGHD, patients. Only one patient from our study had an underdeveloped optic nerve; the other four patients had ocular malformation related to septo-optic dysplasia (SOD). It is remarkable that the OTX2 mutation happened to be present in the only patient with an underdeveloped optic nerve and not among the four patients with other SOD-related ocular malformation.

The Pro134Arg mutation is located outside the DNA binding domains of the OTX2 protein; therefore, as would be expected, no alterations in binding affinity would be predicted. Indeed, EMSA showed that binding to the consensus site by the mutant protein was similar to that of the WT protein (Fig. 2A). Functional expression studies demonstrate that the OTX2 Pro134Arg mutant protein inhibited target gene expression in a dominant negative fashion using a

luciferase reporter fused with a multiple bicoid binding site and expressing increasing concentrations of mutant OTX2 protein (Fig. 2B and C). The multiple bicoid binding site is identical to the OTX2 core binding motif described in the Hesx1 promoter, a known target of OTX2 (12, 14). As the mutation is present in the heterologous state, our studies were developed to determine whether dominant negative inhibition was the mechanism for target gene repression. Increased target expression was clearly shown in cells transfected with WT *Otx2*; however, despite keeping the amount of transfected WT *Otx2* DNA constant, the cotransfection of mutant *Otx2* leads to a significant decrease in reporter expression. In the 293T cells, inhibition became more significant with increasing amounts of mutant *Otx2*. Interestingly, the mutant OTX2 demonstrated an even more dramatic inhibition of reporter expression in the GNRH expressing cell line. We believe that this further highlights the importance of this mutation given that our group has recently demonstrated OTX2 mRNA expression in GN cells (data not shown). As others have shown that the GNRH promoter contains the OTX2 binding site and OTX2 is required for GNRH expression (22), our functional studies further support the role for the mutant protein in the pathogenesis of the central hypogonadism in addition to the other pituitary deficiencies seen in our patient.

The complexity of the OTX2 genotype/phenotype relationship is enormous. Our patient's phenotype is severe and can be explained by the dominant negative mutation. However, the father, being a carrier of the same mutation, should have at least some phenotypic anomalies. Further *in vivo* modeling studies would be required to find the mechanism and other possible factors that could protect from the severe effect of dominant negative inhibition.

To date, 29 different OTX2 mutations have been reported in 32 unrelated patients, including the new missense mutation found in our study (Table 2, Figs 3 and 4). The first described genetic defects that affected the OTX2 gene were interstitial deletions at 14q22, in association with AO and pituitary abnormalities (4, 5). Using the candidate gene approach,

Table 1 Clinical data of five patients with hypopituitarism and eye phenotype.

Case	Gender	Hypopituitarism	Ocular lesion	MRI abnormalities	OTX2
1	Male	CPHD	Underdeveloped left optic nerve (blindness of one eye)	Ectopic posterior pituitary, invisible stalk	Mutation p.Pro134Arg
2	Male	CPHD	SOD suspected, low vision	Ectopic posterior pituitary, invisible stalk	Wild-type
3	Male	CPHD	SOD, hypertelorism, strabismus, and epicanthus	Partially empty sella, absent posterior pituitary	Wild-type
4	Female	IGHD	Strabismus divergens alternans, very low vision, and 'aspecific form of SOD'	Small anterior pituitary, ectopic posterior pituitary, very small sella	Wild-type
5	Male	IGHD	Cavum septum pellucidum	Very small anterior pituitary and ectopic posterior pituitary	Wild-type

CPHD, combined pituitary hormone deficiency; IGHD, isolated GH deficiency; SOD, septo-optic dysplasia.

Table 2 OX2 mutations reported till date. Description of mutations is based on the NM_172337.1 reference sequence, followed HGVS recommendations (www.hgvs.org/mutnomen). Position + 1 refers to the A position of the ATG initiation codon for that gene. Nomenclature may differ from the notation used in the original publication. The novel mutation is in bold type.

cDNA	Protein	Functional test	Phenotype	Inheritance	Remarks	References
1 c.81delC	p.Ser28ProfsX23	Yes (totally inactive) (21)	Severe bilateral MO	Maternal gonosomal mosaicism (carrier)	Consanguineous parents; mother had affected fetus (bilateral MO) carrier of the same mutation; maternal polymorphic chromosome variant inv(2)(p11.2; q13) <i>LRP2</i> homozygous deletion c.11469_11472delTTTG (26)	(8)
2 c.85G>A	p.Val29Met	Not done	Donnai-Barrow syndrome (bilateral iris coloboma, cataract, and retinal abnormalities)	Father carrier (no phenotype)		(6)
3 c.93C>G	p.Tyr31X	Not done	Left MO and right nystagmus	<i>De novo</i>		(6)
4 c.106dupC	p.Arg36ProfsX52	Not done	Siblings: i) right MO and ii) right AO and left coloboma	<i>De novo</i>	Both parents are wild-type (gonosomal parental mosaicism?)	(6)
5 c.117_118delCC	p.Arg40GlyfsX47	Yes (totally inactive) (21)	Bilateral AO, small optic nerves, thin chiasm; hypotelorism; DD	Mother mutation carrier (no phenotype)	Small stature	(8)
6 c.136dupA	p.Thr46AsnfsX42	Not done	i) Bilateral MO and severe optic nerve hypoplasia and ii) father: unilateral MO, cataract, optic nerve aplasia, and anterior segment dysgenesis	Affected father carrier	Caucasian, i) sacral dimple and anteriorly placed anus	(9)
7 c.214_217del GCACinsCA	p.Ala72HisfsX15	Yes (no nuclear localization and no transactivation)	Bilateral MO, normal stature	<i>De novo</i>	Japanese, 1-year-old at the time of the study	(7)
8 c.221_236del	p.Lys74SerfsX30	Yes (no nuclear localization and no transactivation)	Right AO and left MO, DD, IGHD, pituitary hypoplasia, and retractile testis	<i>De novo</i>	Japanese	(7)
9 c.265C>G	p.Arg89Gly	Yes (weak binding activity and reduced transactivation) (21)	Bilateral MO, optic nerve aplasia, absent chiasm	<i>De novo</i>	Cognitive and language skills age appropriate; mother had stillborn infant	(8)
10 c.265C>T	p.Arg89X	Not done	Bilateral AO	<i>De novo</i>		(10)
11 c.270A>T	p.Arg90Ser	Yes (inhibited DNA binding and transactivation)	Right unilateral AO, IGHD, pituitary hypoplasia, and ectopic posterior lobe	Father carrier (short, no ocular phenotype)	Jewish family with different ocular phenotype (no genetic data)	(17)
12 c.289C>T	p.Gln97X	Not done	Siblings: i) extreme bilateral MO and ii) right inferior iris coloboma and left retinal coloboma	Father carrier (reduced vision in one eye)	Dizygotic twins	(6)
13 c.295C>T	p.Gln99X	Yes (no transactivation activities) (21)	Bilateral MO, absent optic nerves and chiasm, asymmetry of the lateral ventricles, seizures	Father mutation carrier (no phenotype)	Intracerebral bleed in the newborn period	(8)
14 c.313C>T	p.Gln105X	Not done	Bilateral AO, bilateral dysplastic globes and optic nerve aplasia	<i>De novo</i>	Caucasian, latent Wolf-Parkinson-White syndrome, feeding difficulties	(9)

Table 2 Continued

cDNA	Protein	Functional test	Phenotype	Inheritance	Remarks	References
15 c.373_374delAG	p.Ser125TrpfsX11	Not done	Bilateral AO and mild DD	Mother carrier (no phenotype)		(6)
16 c.397C>A	p.Pro133Thr	Yes (normal) (21)	Right MO and cataract, left sclerocornea	Mother and brother mutation carriers (no phenotype)		(8)
17 c.400C>G	p.Pro134Ala	Yes (normal) (21)	Left AO, early mild DD, attention-deficit/hyperactivity disorder	<i>De novo</i>	Mother with left AO is wild-type; half brother with learning difficulties	(8)
18 c.401C>G	p.Pro134Arg	Yes (dominant negative effect)	CPHD, underdeveloped left optic nerve, pituitary hypoplasia	Father carrier	Dutch, general GH-deficient appearance and severe behavioral problems	This study
19 c.402dupC	p.Ser135LeufsX2	Yes (no transactivation activities)	Bilateral AO, short stature, and partial IGHD; DD	<i>De novo</i>	MRI normal, Japanese	(13)
20 c.404_405dupCT	p.Ser136LeufsX43	Yes (not transactivation HESX1 and POU1F1)	Bilateral AO, CPHD, pituitary hypoplasia, ectopic posterior lobe and chiari malformation, DD	<i>De novo</i>	Japanese	(16)
21 c.413C>G	p.Ser138X	Not done	GHD (low IGf1 and IGFBP3), alternating esotropia with mild torsional nystagmus	<i>De novo</i>	Failure to thrive and feeding	(15)
22 c.456_457delGAinsAT	p.Trp152X	Not done	Right AO and left MO, bilateral optic nerve hypoplasia, absent chiasm; DD	<i>De novo</i>	Caucasian, consanguineous parents, and feeding difficulties	(9)
23 c.463_464dupGC	p.Ser156LeufsX23	Yes (no transactivation activities) (21)	Right AO and left MO, iris and chorioretinal coloboma, total retinal detachment on the left; partial agenesis of corpus callosum, absent right and small left optic nerves, thin chiasm; growth delay; DD	<i>De novo</i>	Mother had hypopigmented macula and fundus; normal pituitary MRI and function	(8)
24 c.532A>T	p.Thr178Ser	Yes (normal transactivation)	CPHD	Parents refused molecular analysis	Japanese, without ocular anomalies	(7)
25 c.537T>A	p.Tyr179X	Yes (no transactivation activities) (21)	Siblings: i) bilateral MO and colobomata; severe DD and seizures, ii) LCA, bilateral peripheral anterior synchiae iii) MO, aniridia, microphaly, and café au lait macules	Maternal gonosomal mosaicism (with pigmentary retinopathy)	i) Has bilateral fifth finger clinodactyly and ii) has unilateral right-sided hearing loss	(8)
26 c.553_556dupTATA	p.Ser186IlefsX2	Not done	Bilateral MO, hypoplastic optic nerves, small optic chiasm; pituitary hypoplasia; microcephaly, and DD	Father with LCA	Both with small stature (?) Also missense mutation <i>PAX6</i> p.R38W and nonsense mutation <i>NF1</i> p.R192X	(25)
27 c.562G>T	p.Gly188X	Yes (50% reduced transactivation)	i) Bilateral MO, CPHD, pituitary hypoplasia, and DD ii) Bilateral MO, DD, seizures	Not available	Hispanic; hypoplastic labia minora, hypotonia, failure to thrive	(9)
				Not available	Japanese	(7)
				Not available	Japanese, normal stature and normal MRI	(7)

Table 2 Continued

cDNA	Protein	Functional test	Phenotype	Inheritance	Remarks	References
28 c.674A>G	p.Asn225Ser	Yes (dominant negative effect)	i) CPHD, pituitary hypoplasia ii) CPHD, pituitary hypoplasia	Not reported	Without ocular anomalies	(14)
29 c.734C>T	p.Ala245Val	Yes (normal transactivation)	Bilateral optic nerve hypoplasia and short stature	Father carrier (normal phenotype)	Without ocular anomalies	(14)
Microdeletion chr 14, 46.XX,del(14Xq22q23)	Whole gene	NA	Bilateral AO; absent pituitary, optic nerves, chiasma, and tracts; underdeveloped genitalia and micrognathia	<i>De novo</i>	Japanese	(7)
Microdeletion chr 14, 46.XY,del(14q22.1–q22.3)	Whole gene	NA	Bilateral AO, micrognathia, hypogonadism, hypothyroidism, growth retardation, DD, and dentation delay	<i>De novo</i>	Caucasian; fetus, fourth abortion	
Balanced translocation 46.XY, t(3;14)(q28;q23.2)	Whole gene	NA	AO, IGHD, pituitary hypoplasia, absence of optic nerves, chiasm tracts; ear anomalies; undescended testes; DD	<i>De novo</i>	9.66 Mb deletion, including genes: <i>BMP4</i> , <i>OTX2</i> , <i>RTN1</i> , <i>SIX6</i> , <i>SIX1</i> , and <i>SIX4</i> , syndactyly and brachydactyly	(23)
Microdeletion Chr 14:53758044–56834649	Whole gene	NA	Bilateral extreme MO	<i>De novo</i>	3.07 Mb deletion	(6)
Microdeletion Chr14:56268037–57541514	Whole gene	NA	Bilateral AO, DD (maybe due to head trauma)	<i>De novo</i>	1.28 Mb deletion	(6)
Microdeletion Chr14:56006531–58867091	Whole gene	NA	Right MO and left AO; DD, IGHD and pituitary hypoplasia	<i>De novo</i>	Japanese; 2.9 Mb deletion +931 bp addition, other 16 genes deleted	(7)
(NC_000014.7) Duplication of 14q22.3–q23.3 insertion in 13q21	Whole gene	NA	Branchiootorenal syndrome and oculoauriculovertebral spectrum: growth delay, microcephaly, micrognathia, right-sided optic nerve hypoplasia, hearing loss, significant DD	Father	Father and two other family members with same aberration and DD	(24)

OTX2, orthodenticle *Drosophila* homolog 2; AO, anophthalmia; MO, microphthalmia; LCA, Leber congenital amaurosis; DD, developmental delay; IGHD, isolated GH deficiency; CPHD, combined pituitary hormone deficiency; NA, not applicable.

Figure 3 OTX2 structure and mutation mapping. Genomic and protein structure of human OTX2 major isoform b (NM_172337.1; NP_758840.1). Protein domains are defined as described in Chatelain *et al.* (21) and the conserved domains. HD, homeobox domain; NRS, nuclear retention signal; OTX, OTX family domain; orange box represents the SIWSPA conserved motif; the closed triangles represent two tandem repeated conserved transactivation motifs. OTX2 mutations in patients described with only eye malformation are indicated in black boxes, with additional pituitary malformations in green boxes, and exclusively with pituitary malformations in blue boxes. The novel mutation is presented in bold. Horizontal triangles represent the gradient of the eye and pituitary phenotypes described in the patients with OTX2 mutations.

Ragge *et al.* (8) screened a large cohort of 333 patients with AO, MO, and/or coloboma identifying the first eight mutations. In the following years, several other studies performed OTX2 gene sequencing in patients with severe ocular malformations (6, 9).

HESX1 is an important transcription factor involved in pituitary development. HESX1 has been shown to be a transcriptional target of OTX2 (11, 12); thus, our current screening of OTX2 was a logical step following our previous search for HESX1 mutations (19).

The pathogenicity of the OTX2 mutations is not easy to interpret, although several studies performed a detailed analysis of the biological effects of these mutations (7, 13, 14, 21). The inheritance pattern of the OTX2 mutations is usually described as autosomal recessive with variable phenotype. Incomplete penetrance has been described in many families (6, 7, 8, 17). This would explain the existence of unaffected carriers, like the father of our patient.

A relatively large number of alterations were found to be *de novo*, especially in all cases with whole gene deletions and translocations (4, 5, 6, 7, 8, 13, 16, 23). The remarkable exception to this is the duplication of 14q22.3 (24) that was inherited from the affected father and was present in several family members with less severe developmental delay. However, some cases add more complexity to this pattern, like two cases of gonosomal mosaicism inheritance described by Ragge *et al.* (8) (Table 2: mutations 1, 25a and b). Moreover, Wyatt *et al.* (6) describe a family where normal parents without mutations had two affected children

with the same mutation but different phenotypes (Table 2: mutation 4); parental mosaicism was excluded in blood and buccal cells. In many cases, one or several relatives are carriers of the same mutation and have apparently normal or mild phenotype (Table 2: mutations 2, 5, 11, 12, 13, 15, 16, and 29). In this regard, it should be noted that all mutations and deletions found to date are strictly heterozygous, although several cases with additional mutations in other genes have been described (6, 25) (Table 2: mutations 2 and 25c). The above-mentioned cases suggest the existence of other genetic or environmental factors that influence the phenotype of patients with OTX2 mutations. Similar phenomena are seen in mouse models; for example, the *Otx2*-null mice have a severe head phenotype, but heterozygous animals have a variable phenotype ranging from apparent normality to severe developmental eye and head abnormalities depending on genetic background (1, 2, 3).

It is known that prenatal exposure to teratogens can cause eye malformation; therefore, environmental factors may also play a role in the phenotype and explain some of the differences between individuals with the same mutation. In at least two cases, the mothers of the patients reported exposure to probably damaging factors during pregnancy (8) (Table 2: mutations 1 and 13).

In order to draw global conclusions from the mutational analyses performed to date, we summarized the known 29 OTX2 mutations together with the reported phenotypic data in Table 2 and Figs 2 and 3. Mutations within OTX2 are found throughout the

NP_758840.1 OTX2_b_[Homo_sapie	MMSYLKQ--PPYAVNGLSLTSGMDLLHPSVGY P -----ATP R KQ R RE 41
NP_068374.1 OTX2_a_[Homo_sapie	MMSYLKQ--PPYAVNGLSLTSGMDLLHPSVGYPGPWASCPAATPRKQ R RE 49
NP_001171116.1 Otx2[Macaca_mul	MMSYLKQ--PPYAVNGLSLTSGMDLLHPSVGYPGPWASCPAATPRKQ R RE 49
NP_659090.1 Otx2[Mus_musculus]	MMSYLKQ--PPYAVNGLSLTSGMDLLHPSVGY P -----ATP R KQ R RE 41
NP_571326.1 otx2[Danio_rerio]	MMSYLKQ--PPYAVNGLSLTSGMDLLHPSVGY P -----ATP R KQ R RE 41
NP_001186699.1 OTX1[Homo_sapie	MMSYLKQ--PPYGMNGLGLAGPAMDLLHPSVGY P -----ATP R KQ R RE 41
NP_000545.1 CRX[Homo_sapiens]	MMAYMNP G PHYSVNALALSGPSVDLMHQAVPY P -----SAP R KQ R RE 42
	:: * * :*:***: ..:***: * * ** :*:*****
NP_758840.1 OTX2_b_[Homo_sapie	RTTF T RAQLDVL E ALFAKTRYPDIFMREEV A LKINLPESRVQVWFKN R RA 91
NP_068374.1 OTX2_a_[Homo_sapie	RTTFTRAQLDVL E ALFAKTRYPDIFMREEV A LKINLPESRVQVWFKN R RA 99
NP_001171116.1 Otx2[Macaca_mul	RTTFTRAQLDVL E ALFAKTRYPDIFMREEV A LKINLPESRVQVWFKN R RA 99
NP_659090.1 Otx2[Mus_musculus]	RTTFTRAQLDVL E ALFAKTRYPDIFMREEV A LKINLPESRVQVWFKN R RA 91
NP_571326.1 otx2[Danio_rerio]	RTTFTRAQLDVL E ALFAKTRYPDIFMREEV A LKINLPESRVQVWFKN R RA 91
NP_001186699.1 OTX1[Homo_sapie	RTTFTRSQLDVL E ALFAKTRYPDIFMREEV A LKINLPESRVQVWFKN R RA 91
NP_000545.1 CRX[Homo_sapiens]	RTTFTRSQLEEL E ALFAKTQYPDVYAREEVALKINLPESRVQVWFKN R RA 92
	*****:***: *****:***: *****:*****:*****:*****
NP_758840.1 OTX2_b_[Homo_sapie	KCR----QQ Q QQQNGGQNKVRPAKKKT--SP---AREVSSESGTSGQF 131
NP_068374.1 OTX2_a_[Homo_sapie	KCR----QQQQQQQNGGQNKVRPAKKKT--SP---AREVSSESGTSGQF 139
NP_001171116.1 Otx2[Macaca_mul	KCR----QQQQQQQNGGQNKVRPAKKKT--SP---AREVSSESGTSGQF 139
NP_659090.1 Otx2[Mus_musculus]	KCR----QQQQQQQNGGQNKVRPAKKKS--SP---AREVSSESGTSGQF 131
NP_571326.1 otx2[Danio_rerio]	KCR----QQQQQQQNGGQNKVRPAKKKS--SP---AREVSSESGTSGQF 131
NP_001186699.1 OTX1[Homo_sapie	KCR----QQQQ--SGSGTKSRPAKKKS--SP---VRESSG-SESSGQF 127
NP_000545.1 CRX[Homo_sapiens]	KCRQQRQQQKQQQPPGGQAKARPAK R KAGTS P RPST D VC P DLGISDSY 142
	*** :*: * . * *****: * * . . . * .. :
NP_758840.1 OTX2_b_[Homo_sapie	TPPSSTSVPTIASSS-----APVSI W 152
NP_068374.1 OTX2_a_[Homo_sapie	TPPSSTSVPTIASSS-----APVSIW 160
NP_001171116.1 Otx2[Macaca_mul	TPPDSTSVPTIASSS-----AHGSIW 160
NP_659090.1 Otx2[Mus_musculus]	SPPSSTSVPTIASSS-----APVSIW 152
NP_571326.1 otx2[Danio_rerio]	TPPSSTSVPAISTTT-----APVSIW 152
NP_001186699.1 OTX1[Homo_sapie	TPPAVSSSSASSSSASSSSANPAAAAAGLG N PNVAAASSLSTPAASSIW 177
NP_000545.1 CRX[Homo_sapiens]	SPPLPGSPSGSPPTAV-----ATVSIW 163
	:** . : : : : * **
NP_758840.1 OTX2_b_[Homo_sapie	SPASIS P -----LSDPLSTSS--SCMQ R -----SYPM T YTQ 181
NP_068374.1 OTX2_a_[Homo_sapie	SPASIS P -----LSDPLSTSS--SCMQ R -----SYPM T YTQ 189
NP_001171116.1 Otx2[Macaca_mul	SPASIS P -----LSDPLSTSS--SCMQ R -----SYPM T YTQ 189
NP_659090.1 Otx2[Mus_musculus]	SPASIS P -----LSDPLSTSS--SCMQ R -----SYPM T YTQ 181
NP_571326.1 otx2[Danio_rerio]	SPASIS P -----LSDPLSTSS--SCMQ R -----SYPM T YTQ 181
NP_001186699.1 OTX1[Homo_sapie	SPASISPGSAPASVSVPEPLAAPSNTSCMQRSVAAGAATAAASYPMSY G Q 227
NP_000545.1 CRX[Homo_sapiens]	SPASESP-----LPEAQRAGLVASGPSLTS-----APYAM T YAP 197
	**** * : : . : * . . * . : *
NP_758840.1 OTX2_b_[Homo_sapie	ASGY S ---QGYAG-STSYFGGMDCGSYLTPMH H QLPGPGATLSPMG T NAV 227
NP_068374.1 OTX2_a_[Homo_sapie	ASGYS---QGYAG-STSYFGGMDCGSYLTPMH H QLPGPGATLSPMG T NAV 235
NP_001171116.1 Otx2[Macaca_mul	ASGYS---QGYAG-STSYFGGMDCGSYLTPMH H QLPGPGATLSPMG T NAV 235
NP_659090.1 Otx2[Mus_musculus]	ASGYS---QGYAG-STSYFGGMDCGSYLTPMH H QLPGPGATLSPMG T NAV 227
NP_571326.1 otx2[Danio_rerio]	ASGYS---QGYAG-STSYFGGMDCGSYLTPMH H QLTGPSTLSPMSS N AV 227
NP_001186699.1 OTX1[Homo_sapie	GGSYG---QGYTPSSSYFGGVDCSSYLAPMHSHHH P --HQLSPMAPSSM 272
NP_000545.1 CRX[Homo_sapiens]	ASAFCSPPSAYGS-PSSYFSGGLD P --YLS P MPVQLG G --PALSLSPGSPV 242
	...: . * . :***:***: **:* : ***: . : :
NP_758840.1 OTX2_b_[Homo_sapie	TSHLNQS-----PASLST-----QGYG A SSLGFNSTTDCLDYKD 261
NP_068374.1 OTX2_a_[Homo_sapie	TSHLNQS-----PASLST-----QGYGASSLGFNSTTDCLDYKD 269
NP_001171116.1 Otx2[Macaca_mul	TSHLNQS-----PASLST-----QGYGASSLGFNSTTDCLDYKD 269
NP_659090.1 Otx2[Mus_musculus]	TSHLNQS-----PASLST-----QGYGASSLGFNSTTDCLDYKD 261
NP_571326.1 otx2[Danio_rerio]	TSHLNQS-----PASLPT-----QGYGASSLGFNSTADCLDYKD 261
NP_001186699.1 OTX1[Homo_sapie	AGHHHHHPHAHHPLSQSSGHHHHHHHHHHQGYGSGGLAFNS-ADCLDYKE 321
NP_000545.1 CRX[Homo_sapiens]	GPSLAQS-----PTLSG-----QSYGAY-----SPVDSLEFKD 271
	: * * . * . * . * . * . : *
NP_758840.1 OTX2_b_[Homo_sapie	-----QTASWKLNFN-ADCLDYKDQTSSWKFQVL 289
NP_068374.1 OTX2_a_[Homo_sapie	-----QTASWKLNFN-ADCLDYKDQTSSWKFQVL 297
NP_001171116.1 Otx2[Macaca_mul	-----QTASWKLNFN-ADCLDYKDQTSSWKFQVL 297
NP_659090.1 Otx2[Mus_musculus]	-----QTASWKLNFN-ADCLDYKDQTSSWKFQVL 289
NP_571326.1 otx2[Danio_rerio]	-----QASSWKLNFN-ADCLDYKDQTSSWKFQVL 289
NP_001186699.1 OTX1[Homo_sapie	PGAAAASSAWKLNFNSPDCLDYKDQAS-WRFQVL 354
NP_000545.1 CRX[Homo_sapiens]	-----PTGTWKTFTYNPMDPLDYKDQSA-WKFQIL 299
	: : * : : * * *****: * : * :

Figure 4 Conservation annotation of known mutations in *OTX2* and related proteins. A Clustal W2-based multiple alignment of *OTX2* human isoforms a and b, orthologs from *Macaca mulata*, *Mus musculus*, and *Danio rerio* and paralogs *OTX1* and *CRX* proteins. Homeobox and SIWSPA domains are marked with upper line. Protein changes are highlighted in gray and bold (only, missense), italic (nonsense), and underline (frameshift deletion or insertions).

gene, affecting all three coding exons. However, the distribution seems to have several hotspots affecting functional domains like the homeobox, the nuclear retention signal, or the highly conserved SIWSPA peptide sequence ('SIWSPA conserved motif', Fig. 4). There are several mutations affecting the same amino acid, in various unrelated patients with different phenotypes (Table 2: mutations 9 and 10; 17 and 18; 25, 27, 28). Most mutations are located in conserved residues (Fig. 4), which suggest functional relevance. However, their importance is not always functionally proven.

There seems to be a clear relationship between the localization of the reported mutations and clinical phenotype. Mutations in the N-terminus show very severe eye malformations without pituitary phenotype, while mutations localized to the C-terminus are associated with pituitary malformation and CPHD. All patients reported to date with CPHD, IGHD, or short stature have their mutations in exon 5, except for p.Lys74SerfsX30 (K74fs in Fig. 3). Although many patients have growth retardation or developmental problems during childhood, most of them are born with weights and lengths within the normal range. Patients with CPHD, IGHD, or short stature are overrepresented in the nuclear retention signal and OTX family domain (Fig. 3). Additional research is needed to provide better understanding of the different functional regions of OTX2.

Another conclusion that can be drawn when reviewing the literature is that alterations in the OTX2 affect both genders equally; furthermore, there does not seem to be any relationship between the localization of the mutations and their inheritance (paternal, maternal inheritance, or *de novo*).

Although several investigators have already implicated mechanisms and potential additional factors by which OTX2 mutations can cause the associated phenotype, further research is needed to more clearly define the role of OTX2 in pituitary pathology. Our current studies provide further support for the important role of OTX2 as a candidate gene in genetic screening in patients with CPHD. In addition, we provide further evidence regarding the association between mutations in the C-terminus of the OTX2 gene and clinical presentation of pituitary abnormalities, therefore emphasizing the need for a careful evaluation of the genetic sequence in this area of the OTX2 gene.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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