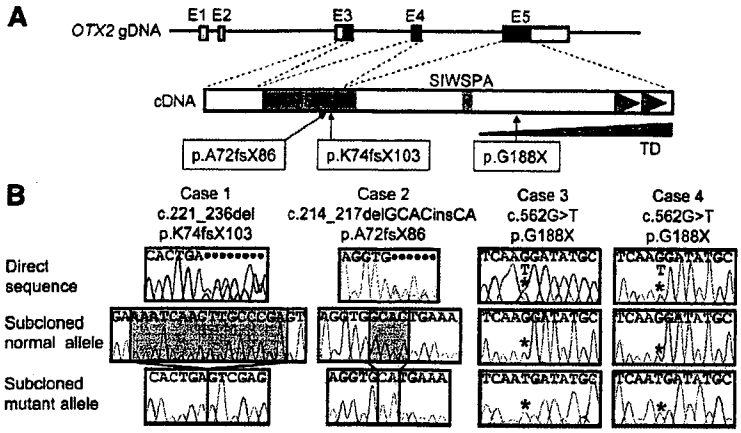

FIG. 1. Sequence analysis in cases 1–4. A, The structure of *OTX2* (the isoform-b) and the position of the mutations identified. The *black* and *white boxes* on genomic DNA (gDNA) denote the coding regions on exons 1-5 (E1-E5) and the untranslated regions, respectively. *OTX2* encodes the HD (a *blue region*), the SIWSPA conserved motif (an *orange region*), and the two tandem tail motifs (*green triangles*). The TD (a *gray triangle*) is assigned to the C-terminal side; deletion of each tail motif reduces the transactivation function, and that of a region distal to the SIWSPA motif further reduces the transactivation function. In addition, another TD may also reside in the 5′ side of the HD (17). The three mutations identified in this study are shown. B, Electrochromatograms showing the mutations in cases 1–4. Shown are the direct sequences and subcloned normal and mutant sequences. The deleted sequences are shaded in *gray*, and the inserted sequence is highlighted in *yellow*. The mutant and the corresponding wild-type nucleotides are indicated by *red asterisks*.

detected by avidin conjugated to fluorescein isothiocyanate. To indicate an extent of a microdeletion, oligoarray comparative genomic hybridization (CGH) was carried out with 1×244K human genome array (catalog no. G4411B; Agilent Technologies, Palo Alto, CA), according to the manufacturer's protocol. Finally, to characterize a microdeletion, long PCR was performed with primer pairs flanking the deleted region, and a long PCR product was subjected to direct sequencing using serial sequence primers. The deletion size and the junction structure were determined by comparing the obtained sequences with the reference sequences at the National Center for Biotechnology Information Database (NC_000014.7; Bethesda, MD), and the presence or absence of repeat sequences around the breakpoints was examined with Repeatmasker (http://www.repeatmasker.org).

Functional studies

Western blot analysis, subcellular localization analysis, DNA binding analysis, and transactivation analysis were performed by the previously reported methods (8) (for details, see Supplemental Methods). In this study, we used the previously reported expression vector and fluorescent vector containing the wild-type *OTX2* cDNA; the probes with the wild-type and mutated OTX2 binding sites within the *IRBP*, *HESX1*, and *POU1F1* promoter sequences; and the luciferase reporter vectors containing the *IRBP*, *HESX1*, and *POU1F1* promoter sequences (8). We further created expression vectors and fluorescent vectors containing mutant *OTX2* cDNAs by site-directed mutagenesis using Prime STAR mutagenesis basal kit (Takara, Otsu, Japan), and constructed a 30-bp probe with wild-type (TAATCT) and mutated (TGGGCT) putative OTX2 binding site within the *GNRH1* promoter sequence and a luciferase reporter vector containing the *GNRH1* promoter sequence (−1349 to −1132 bp) by inserting the corresponding sequence into pGL3 basic. The *GNRH1* promoter sequence was based on the report of Kelley *et al.* (15). Transfections were performed in triplicate within a single experiment, and the experiment was repeated three times.

PCR-based expression analysis of *OTX2*

Human cDNA samples were purchased from CLONTECH (Palo, Alto, CA) except for leukocyte and skin fibroblast cDNA samples that were prepared with Superscript III reverse transcriptase (Invitrogen). PCR amplification was performed for the cDNA samples (0.5 ng), using the primers hybridizing to exon 3 and 4 of *OTX2* and those hybridizing to exons 2/3 and 4/5 (boundaries) of *GAPDH* used as an internal control.

Results

Identification of mutations and substitutions

Three novel heterozygous *OTX2* mutations were identified in four cases, *i.e.* a 16-bp deletion at exon 4 that is predicted to cause a frameshift at the 74th codon for lysine and resultant termination at the 103rd codon (c.221_236del16, p.K74fsX103) in case 1; a 4-bp deletion and a 2-bp insertion at exon 4 that is predicted to cause a frame shift at the 72nd codon for alanine and resultant termination at the 86th codon (c.214_217delGCACinsCA, p.A72fsX86) in case 2; and a nonsense mutation at exon 5 that is predicted to cause a substitution of the 188th glycine with stop codon (c.562G>T, p.G188X) in two unrelated cases (3 and 4; Fig. 1). In addition, heterozygous missense substitutions were identified in patient 1 (c.532A>T, p.T178S) and patient 2 (c.734C>T, p.A245V). Cases 1 and 3 were from group 1, cases 2 and 4 and patient 2 were from group 2, and patient 1 was from group 3. Parental analysis indicated that frameshift mutations in cases 1 and 2 were absent from the parents (*de novo* mutations), whereas the missense substitution of patient 2 was inherited from phenotypically normal father. The parents of cases 3 and 4 and patient 1 refused molecular studies. All the mutations and the missense substitutions were absent from 100 control subjects.

Prediction of the occurrence of aberrant splicing and NMD

The two frameshift mutations and the nonsense mutation were predicted to influence neither exonic splice enhancers nor splice donor and acceptor sites (Supplemental Tables 2 and 3). Furthermore, the two frameshift mutations were predicted to produce the premature termination codons on the mRNA transcribed from the last exon

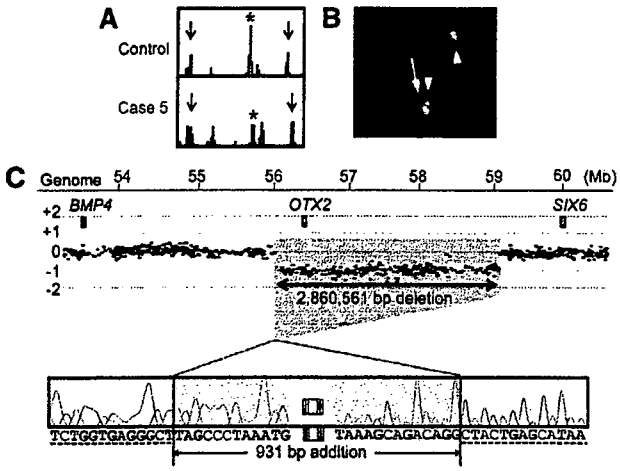

FIG. 2. Deletion analysis in case 5. A, MLPA analysis. The *red asterisk* indicates peaks for the *OTX2* exon 4, and the *black arrows* indicate control peaks. The *red peaks* indicate the internal size markers. Deletion of the MLPA probe binding site is indicated by the reduced peak height. B, FISH analysis. The probe for *OTX2* detects only a *single red signal* (an *arrow*), whereas the RP11-566I2 BAC probe identifies *two green signals* (*arrowheads*). C, Oligoarray CGH analysis and direct sequencing of the deletion junction. The deletion is 2,860,561 bp in physical size (shaded in *gray*) and is associated with an addition of a 931-bp segment (highlighted in *yellow*). The normal sequences flanking the microdeletion are indicated with *dashed underlines*.

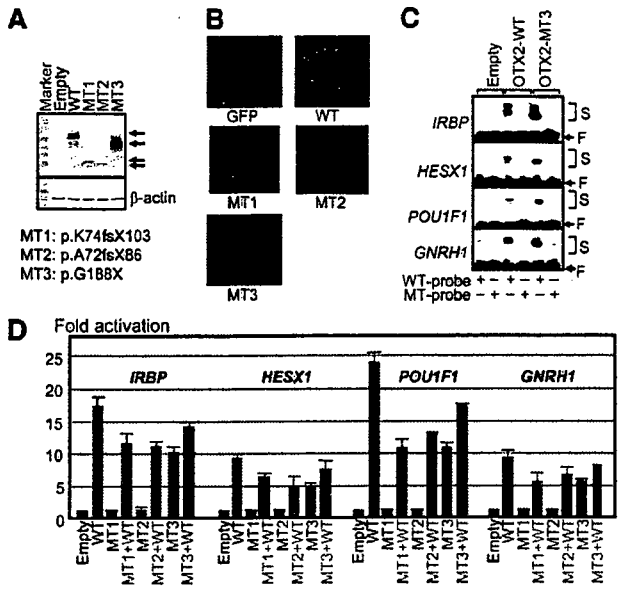

FIG. 3. Functional studies. A, Western blot analysis. Both WT and MT1-MT3 OTX2 proteins are detected with different molecular masses (*arrows*). WT, Wild type; MT1, p.K74fsX103; MT2, p.A72fsX86; and MT3, p.G188X. B, Subcellular localization analysis. Whereas green fluorescent protein (GFP) alone is diffusely distributed throughout the cell, the GFP-fused WT-OTX2 and MT3-OTX2 proteins localize to the nucleus. By contrast, the GFP-fused MT1-OTX2 and MT2-OTX2 proteins are incapable of localizing to the nucleus. C, DNA binding analysis using the wild-type (WT) and mutated (MT) probes derived from the promoters of *IRBP*, *HESX1*, *POU1F1*, and *GNRH1*. The symbols (+) and (–) indicate the presence and absence of the corresponding probes, respectively. Both WT and MT3 OTX2 proteins bind to the WT but not the MT probes. For the probe derived from the *IRBP* promoter, two shifted bands are found for both WT-OTX2 and MT3-OTX2 proteins as reported previously (17). S, Shifted bands; F, free probes. D, Transactivation analysis, using the promoter sequences of *IPBP*, *HESX1*, *POU1F1*, and *GNRH1*. The results are expressed using the mean and SD. The *black*, *blue*, *red*, and *green bars* indicate the data of the empty expression vectors (0.6 μg), expression vectors with WT *OTX2* cDNA (0.6 μg), expression vectors with MT1-MT3 *OTX2* cDNAs (0.6 μg), and the mixture of expression vectors with WT (0.3 μg) and those with MT1-MT3 *OTX2* cDNAs (0.3 μg), respectively; thus, the same amount of expression vectors has been used for each assay.

5, indicating that the frameshift mutations as well as the nonsense mutation had the property to escape NMD (Supplemental Fig. 1).

Identification of a microdeletion

A heterozygous microdeletion affecting *OTX2* was indicated by MLPA and confirmed by FISH in case 5 of group 1 (Fig. 2, A and B). Oligoarray CGH delineated an approximately 2.9-Mb deletion, and sequencing of the fusion point showed that the microdeletion was 2,860,561 bp in physical size (56,006,531–58,867,091 bp on the NC_000014.7) and was associated with an addition of a complex 931-bp segment consisting of the following structures (cen → tel): 2 bp (TA) insertion → 895 bp sequence identical with that in a region just centromeric to the microdeletion (55, 911, 347–55, 912, 241 bp) → 1 bp (C) insertion → 33-bp sequence identical with that within the deleted region (58, 749, 744–58, 749, 776 bp) (Fig. 2C). Repeat sequences were absent around the break points. This microdeletion was not detected in DNA from the parents.

Functional studies of the wild-type and mutant OTX2 proteins

Western blot analysis detected wild-type OTX2 protein of 31.6 kDa and mutant OTX2 proteins of 11.5 kDa (p.K74fsX103), 9.7 kDa (p.A72fsX86), and 15.4 kDa (p.G188X) (Fig. 3A). The molecular masses were as predicted from the mutations. The band intensity was comparable between the wild-type OTX2 protein and the p.G188X-OTX2 protein and was faint for the p.K74fsX103-OTX2 and p.A72fsX86-OTX2 proteins.

Subcellular localization analysis showed that the p.G188X-OTX2 protein localized to the nucleus as did the wild-type OTX2 protein, whereas the p.K74fsX103-OTX2 and p.A72fsX86-OTX2 proteins were incapable of localizing to the nucleus (Fig. 3B). The results were consistent with those of the Western blot analysis because nuclear extracts were used for the Western blotting, with some probable contamination of cytoplasm.

DNA binding analysis revealed that the p.G188X-OTX2 protein with nuclear localizing capacity bound to the wild-type OTX2 binding sites within the four promoters examined, including the *GNRH1* promoter, but not to the mutated OTX2 binding sites (Fig. 3C). The band shift

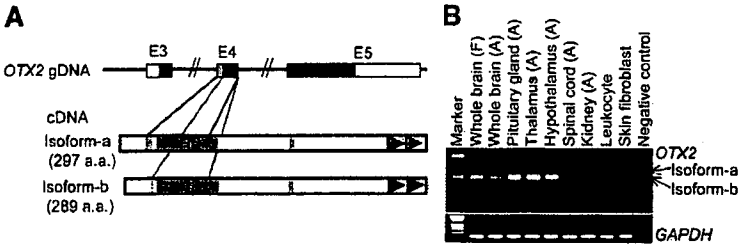

FIG. 4. PCR-based human cDNA library screening for *OTX2* (35 cycles). A, Schematic representation of the *OTX2* isoform-a (NM_21728.2) and isoform-b (NM_172337.1). Because of the two alternative splice acceptor sites at the boundary between intron 3 and exon 4, isoform-a carries eight amino acids (shown in *gray*) in the vicinity of the HD, whereas isoform-b is lacking the eight amino acids. B, PCR amplification data. *OTX2* is clearly expressed in the pituitary and hypothalamus, with isoform-b being the major product. *GAPDH* has been used as an internal control. F, Fetus; A, adult.

was more obvious for the wild-type OTX2 protein than for the p.G188X-OTX2 protein, consistent with the difference in the molecular masses.

Transactivation analysis showed that the wild-type OTX2 protein had transactivation activities for the four promoters examined including the *GNRH1* promoter, whereas the p.K74fsX103-OTX2 and p.A72fsX86-OTX2 proteins had virtually no transactivation function, and the p.G188X-OTX2 protein had reduced (~50%) transactivation activities (Fig. 3D). The three mutant OTX2 proteins had no dominant-negative effects. In addition, the two missense p.A245V-OTX2 and p.T178S-OTX2 proteins had apparently normal transactivation activities with no dominant-negative effect (Supplemental Fig. 2).

PCR-based expression analysis of *OTX2*

OTX2 expression was identified in the pituitary and the hypothalamus as well as in the brain and the thalamus but not detected in the spinal cord, kidney, leukocytes, and skin fibroblasts (Fig. 4). The isoform-b lacking the eight amino acids was predominantly expressed.

Clinical findings in *OTX2* mutation-positive patients

Clinical data are summarized in Table 1 (*left part*). Anophthalmia and/or microphthalmia was present in cases 1–5. Developmental delay was obvious in cases 1 and 3–5, whereas it was obscure in case 2 because of the young age. Prenatal growth was normally preserved in cases 1–5, whereas postnatal growth was compromised in cases 1, 3, and 5. Cases 1 and 5 had IGHD, and case 3 had CPHD (Table 2); furthermore, cases 1, 3, and 5 had pituitary hypoplasia (PH) and/or ectopic posterior pituitary (EPP) (Supplemental Fig. 3). Case 3 showed no pubertal development at 15 yr of age (Tanner pubic hair stage 2 in Japanese boys: 12.5 ± 0.9 yr) (16). Cases 2 and 4 had no discernible pituitary dysfunction and did not receive magnetic resonance imaging examinations. In addition, case 1 had right retractile testis. Patient 1 with p.T178S had CPHD but without ocular anomalies, and patient 2 with p.A245V had bilateral optic nerve hypoplasia and short stature.

Discussion

We identified two frameshift mutations in cases 1 and 2 and a nonsense mutation in unrelated cases 3 and 4. Furthermore, it was predicted that these mutations neither affected splice patterns nor underwent NMD, although direct analysis using mRNA was impossible due to lack of detectable *OTX2* expression in already collected leukocytes as well as skin fibroblasts, which might be available from cases 1–4. Thus, these mutations are predicted to produce aberrant OTX2 proteins *in vivo* that were used in the *in vitro* functional studies. In this context, the functional studies indicated that the two frameshift mutations were amorphic and the nonsense mutation was hypomorphic. The results are consistent with the previous notion that the HD not only has DNA binding capacity but also retains at least a part of nuclear localization signal on its C-terminal portion and the TD primarily resides in the C-terminal region (17) (Fig. 1A). Whereas the two missense substitutions were absent in 100 control subjects, they would be rare normal variations rather than pathological mutations because of the normal transactivation activities with no dominant-negative effect.

We also detected a heterozygous microdeletion involving *OTX2* in case 5 that was not mediated by repeat sequences. This implies the importance of the examination of a microdeletion. Indeed, such a cryptic microdeletion has been identified in multiple genes with the development of MLPA that can serve as a screening method in the detection of microdeletions (18). Whereas the microdeletion of case 5 has removed 16 additional genes (Ensembl Genome Browser, http://www.ensembl.org/), the clinical phenotype of case 5 is explainable by *OTX2* haploinsufficiency alone. Thus, hemizygosity for the 16 genes would not have a major clinical effect, if any.

Furthermore, the present study revealed two findings. First, *OTX2* was expressed in the hypothalamus and had a transactivation function for the *GNRH1* promoter. This implies that *GNRH1* essential for the hypothalamic GnRH secretion is also a target gene of *OTX2*, as has been demonstrated in the mouse (15). Second, the short isoform-b was predominantly identified in the *OTX2* expression-positive tissues. This sug-

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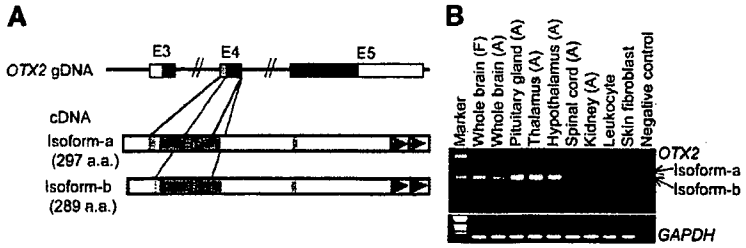
	Present study					Previous studies[a]			
	Case 1	**Case 2**	**Case 3**	**Case 4**	**Case 5**	**Case 6**	**Case 7**	**Case 8**	**Case 9**
Present age (yr)	3	1	15	10	2	3	6	14	6
Sex	Male	Female	Male	Male	Male	Female	Male	Female	Male
Mutation[b]									
cDNA	c.221_236del	c.214_217del GCACinsCA	c.562G>T	c.562G>T	Whole gene deletion	c.402_403insC	c.674A>G	c.674A>G	c.405_406insCT
Protein	p.K74fsX103	p.A72fsX86	p.G188X	p.G188X	Absent	p. S135fsX136	p.N225S	p.N225S	p.S136fsX178
Function	Severe LOF	Severe LOF	Mild LOF	Mild LOF	Absent	Severe LOF	DN	DN	Severe LOF
Ocular malformation									
Right	AO	MO	MO	MO	MO	AO	N.D.	N.D.	AO
Left	MO	MO	MO	MO	AO	AO	N.D.	N.D.	AO
Developmental delay	+	Uncertain	+	+	+	+	N.D.	N.D.	+
Prenatal growth failure[c]	−	−	−	−	−	−	N.D.	N.D.	−
Birth length (cm) (SDS)	46.5 (−1.2)	48.3 (±0)	50 (+0.5)	49 (±0)	47.9 (−0.5)	50 (+0.6)	N.D.	N.D.	49.5 (+0.2)
Birth weight (kg) (SDS)	2.77 (−0.5)	3.22 (+0.6)	3.62 (+1.5)	3.23 (+0.5)	2.96 (−0.1)	3.16 (+0.2)	N.D.	N.D.	3.49 (+1.2)
Birth OFC (cm) (SDS)	32.5 (−0.7)	34 (+0.7)	N.E.	32.5 (−0.7)	31.5 (−1.4)	33.7 (+0.6)	N.D.	N.D.	N.D.
Postnatal growth failure[c]	+	−	+	−	+	+	+	+	+
Present height (cm) (SDS)	76.9 (−3.3)[d]	73.2 (±0)	114.0 (−4.1)[e]	130.8 (−1.5)	78.1 (−2.4)	85.0 (−3.3)	N.D.	N.D.	81.8 (−5.3)[f]
Present weight (kg) (SDS)	8.9 (−2.6)[d]	8.3 (−0.4)	16.8 (−2.4)[e]	23.2 (−1.6)	9.9 (−1.4)	10.1 (−2.6)	N.D.	N.D.	10.7 (−2.5)[f]
Present OFC (cm) (SDS)	N.E.	N.E.	N.E.	N.E.	N.E.	46 (−1.9)	N.D.	N.D.	47.2 (−2.7)[f]
Paternal height (cm) (SDS)[c]	160 (−1.9)	168 (−0.5)	178 (+1.2)	167 (−0.7)	163 (−1.3)	170 (±0)	178 (+0.3)	188 (+1.8)	N.D.
Maternal height (cm) (SDS)[c]	150 (−1.6)	151 (−1.3)	166 (+1.5)	165 (+1.4)	170 (+2.2)	155 (−0.6)	158 (−0.8)	168 (+0.7)	N.D.
Affected pituitary hormones	GH	No	GH, TSH, PRL, LH, FSH	No	GH	GH	GH, TSH, ACTH, LH, FSH	GH, TSH, ACTH, LH, FSH	GH, TSH, ACTH, LH, FSH
MRI findings									
Pituitary hypoplasia	+	N.E.	+	N.E.	+	−	+	+	+
EPP	+	N.E.	+	N.E.	−	−	+	−	+
Other features	Retractile testis (R)			Seizure		Cleft palate			Chiari malformation

SDS, SD score; OFC, occipitofrontal head circumference; MRI, magnetic resonance imaging; LOF, loss of function; DN, dominant negative; AO, anophthalmia; MO, microphthalmia; N.D., not described; N.E., not examined; PRL, prolactin; R, right.

[a] Case 6, Dateki *et al.* (8); cases 7 and 8, Diaczok *et al.* (9); case 9, Tajima *et al.* (10); [b] the cDNA and protein numbes are based on the human *OTX2* isoform-b (GenBank accession no. NM_172337.1), and the A of the ATG encoding the initiator methionine residue is denoted position +1; thus, the description of the mutations in cases 7–9 is different from that reported by Diaczok *et al.* (9) and Tajima *et al.* (10); [c] assessed by the age- and sex-matched Japanese growth standards (27) (cases 1–6 and 9 and their parents) or by the American growth standards (28) (the parents of cases 7 and 8); [d] at 2 yr 4 months of age before GH treatment; [e] at 10 yr of age before GH treatment; [f] at 4 yr of age before GH treatment.



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MRI findings									
Pituitary hypoplasia	+	N.E.	+	N.E.	+	−	+	+	+
EPP	+	N.E.	+	N.E.	−	−	+	−	+
Other features	Retractile testis (R)			Seizure		Cleft palate			Chiari malformation

SDS, SD score; OFC, occipitofrontal head circumference; MRI, magnetic resonance imaging; LOF, loss of function; DN, dominant negative; AO, anophthalmia; MO, microphthalmia; N.D., not described; N.E., not examined; PRL, prolactin; R, right.

[a] Case 6, Dateki *et al.* (8); cases 7 and 8, Diaczok *et al.* (9); case 9, Tajima *et al.* (10); [b] the cDNA and protein numbes are based on the human *OTX2* isoform-b (GenBank accession no. NM_172337.1), and the A of the ATG encoding the initiator methionine residue is denoted position +1; thus, the description of the mutations in cases 7–9 is different from that reported by Diaczok *et al.* (9) and Tajima *et al.* (10); [c] assessed by the age- and sex-matched Japanese growth standards (27) (cases 1–6 and 9 and their parents) or by the American growth standards (28) (the parents of cases 7 and 8); [d] at 2 yr 4 months of age before GH treatment; [e] at 10 yr of age before GH treatment; [f] at 4 yr of age before GH treatment.

TABLE 2. Blood hormone values in cases 1–5 with heterozygous *OTX2* mutations

Patient Sex (age at examination)		Case 1 Male (2 yr)		Case 2 Female (1 yr)		Case 3 Male (14 yr)		Case 4 Male (10 yr)		Case 5 Male (2 yr)	
	Stimulus (dose)	**Basal**	**Peak**	**Basal**	**Peak**	**Basal**	**Peak**	**Basal**	**Peak**	**Basal**	**Peak**
GH (ng/ml)	Insulin (0.1 U/kg)[a]	1.9[b]	**4.0**[b]	3.3[b]	N.E.	0.8[b]	**1.3**[b]	12.1[b]	N.E.	0.5[c]	**9.0**[c]
	Arginine (0.5 g/kg)									1.1[c]	**7.0**[c]
	L-dopa (10 mg/kg)	1.5[b]	**3.8**[b]			0.3[b]	**1.0**[b]				
LH (mIU/ml)	GnRH (100 μg/m^2)	0.1	1.7	0.1	N.E.	2.3[d]	**4.5**	0.4	N.E.	0.1	3.1
FSH (mIU/ml)	GnRH (100 μg/m^2)	1.0	6.2	3.7	N.E.	1.3[d]	**6.3**	1.1	N.E.	1.5	9.9
TSH (μU/ml)	TRH (10 μg/kg)	4.2	23.8	1.1	N.E.	0.2	**1.9**	1.1	N.E.	5.2	19.5
Prolactin (ng/ml)	TRH (10 μg/kg)	17.9	34.5	N.E.	N.E.	5.5	**8.3**	9.1	N.E.	10.43	88.8
ACTH (pg/ml)	Insulin (0.1 U/kg)	31	195	N.E.	N.E.	24		N.E.	N.E.	41	222
Cortisol (μg/dl)[d]	Insulin (0.1 U/kg)	12.7		9.4	N.E.	19.4		N.E.	N.E.	25.4	39.2
IGF-I (ng/ml)		**8**		65	N.E.	**5**		214	N.E.	48	
Testosterone (ng/dl)		N.E.		N.E.	N.E.	**45**		<5	N.E.	N.E.	
Free T_4 (ng/dl)		1.32		1.17	N.E.	**0.87**		1.15	N.E.	1.17	
Free T_3 (pg/ml)		2.91		3.24	N.E.	**1.94**		3.92	N.E.	4.54	

The conversion factor to the SI unit: GH, 1.0 (μg/liter); LH, 1.0 (IU/liter); FSH, 1.0 (IU/liter); TSH, 1.0 (mIU/liter); prolactin, 1.0 (μg /liter); ACTH, 0.22 (pmol/liter); cortisol, 27.59 (nmol/liter); IGF-I, 0.131 (nmol/liter); testosterone, 0.035 (nmol/liter); free T_4, 12.87 (pmol/liter); and free T_3, 1.54 (pmol/liter). Hormone values have been evaluated by the age- and sex-matched Japanese reference data (29, 30); low hormone data are *boldfaced*. Blood sampling during the provocation tests: 0, 30, 60, 90, and 120 min. N.E., Not examined.

[a] Sufficient hypoglycemic stimulations were obtained during all the insulin provocation tests; [b] GH was measured using the recombinant GH standard, and the peak GH values of 6 and 3 ng/ml are used as the cutoff values for partial and severe GH deficiency, respectively; [c] GH was measured by the classic RIA, and the peak GH values of 10 and 5 ng/ml were used as the cutoff values for partial and severe GH deficiency; [d] Obtained at 0800–0900 h.

gests that the biological functions of *OTX2* are primarily contributed by the short isoform-b.

Clinical features of cases 1–5 are summarized in Table 1, together with those of the previously reported *OTX2* mutation-positive patients examined for detailed pituitary function. Here four patients with cytogenetically recognizable deletions involving *OTX2* are not included (19–22) because the deletions appear to have removed a large number of genes including *BMP4* and/or *SIX6* (Fig. 2B) that can be relevant to pituitary development and/or function (1, 23).

Several points are noteworthy for the clinical findings. First, although cases 1–5 in this study had anophthalmia and/or microphthalmia, ocular phenotype has not been described in cases 7 and 8 identified by *OTX2* mutation analysis in 50 patients with hypopituitarism (9). Whereas no description of a phenotype would not necessarily indicate the lack of the phenotype, *OTX2* mutations may specifically affect pituitary function at least in several patients. This would not be unexpected because several *OTX2* mutation-positive patients are free from ocular anomalies (6).

Second, pituitary phenotype is variable and independent of the *in vitro* function data. This would be explained by the notion that haploinsufficiency of developmental genes is usually associated with a wide range of penetrance and expressivity depending on other genetic and environmental factors (24), although the actual underlying factors remain to be identified. In this regard, because direct mRNA analysis was not performed, it might be possible that the mutations have not produced the predicted aberrant protein and, consequently, *in vitro* function data do not necessarily reflect the *in vivo* functions. Even if this is the case, the quite different pituitary phenotype between cases 3 and 4 with the same mutation would argue for the notion that pituitary phenotype is independent of the residual OTX2 function.

Third, cases 1, 3, 5, and 6–9 with pituitary dysfunction have IGHD or CPHD involving GH, and show the combination of preserved prenatal growth and compromised postnatal growth characteristic of GH deficiency (25). This suggests that GH is the most vulnerable pituitary hormone in *OTX2* mutations. Consistent with this, previously reported patients with ocular anomalies and *OTX2* mutations also frequently exhibit short stature (6, 8). Thus, pituitary function studies are recommended in patients with ocular anomalies and postnatal short stature to allow for appropriate hormone therapies including GH treatment for short stature, cortisol supplementation at a stress period, T_4 supplementation to protect the developmental deterioration, and sex steroid supplementation to induce secondary sexual characteristics. Furthermore, *OTX2* mutation analysis is also recommended in such patients.

Lastly, PH and/or EPP is present in patients with IGHD and CPHD, except for case 6 with IGHD. In this regard, the following findings are noteworthy: 1) heterozygous loss-of-function mutations of *HESX1* are associated with a wide phenotypic spectrum including CPHD, IGHD, and apparently normal phenotype and often cause PH and

EPP, whereas homozygous *HESX1* mutations usually lead to CPHD as well as PH and EPP (2); 2) heterozygous loss-of-function mutations of *POU1F1* usually permit apparently normal pituitary phenotype, whereas homozygous loss-of-function mutations and heterozygous dominant-negative mutations usually result in GH, TSH, and prolactin deficiencies and often cause PH but not EPP (2); and 3) heterozygous *GNRH1* frameshift mutation are free from discernible phenotype, whereas homozygous *GNRH1* mutations result in isolated hypogonadotropic hypogonadism with no abnormal pituitary structure (26). Collectively, overall pituitary phenotype may primarily be ascribed to reduced *HESX1* expression, although reduced *POU1F1* and *GNRH1* expressions would also play a certain role, and there may be other target genes of *OTX2*.

In summary, the results imply that *OTX2* mutations are associated with variable pituitary phenotype, with no genotype-phenotype correlations, and that *OTX2* can transactivate *GNRH1* as well as *HESX1* and *POU1F1*. Further studies will serve to clarify the role of *OTX2* in the pituitary development and function.

Acknowledgments

We thank the patients and parents for participating in this study. We also thank Dr. Nicola Ragge and Dr. David J Bunyan for the MLPA probe sequence of *OTX2*.

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This work was supported by Grants-in-Aid for Young Scientists (B-21791025) from the Ministry of Education, Culture, Sports, Science, and Technology and Grants for Child Health and Development (20C-2); Research on Children and Families (H21-005); and Research on Measures for Intractable Diseases (H21-043) from the Ministry of Health, Labor, and Welfare.

Disclosure Summary: The authors have nothing to declare.

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Mutation and Gene Copy Number Analyses of Six Pituitary Transcription Factor Genes in 71 Patients with Combined Pituitary Hormone Deficiency: Identification of a Single Patient with *LHX4* Deletion

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Short title: Dateki et al.; Microdeletion in CPHD patients

Précis: Mutation and gene copy number analyses of six pituitary transcription factor genes in 71 patients reveal only a single female patient with a heterozygous *LHX4* deletion.

Key words: *LHX4*, deletion, MLPA, combined pituitary hormone deficiency

Text 1,728 words, abstract 226 words, references 20, table 1, figure 1, supplemental figures 2, supplemental tables 2, and supplemental figure legends 1.

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Supported by Grant sponsors: Grants for Child Health and Development (20C-2) and Research on Children and Families (H21-005) from the Ministry of Health, Labor, and Welfare, and Grants-in-Aid for Young Scientists (B) (21791025) from the Ministry of Education, Culture, Sports, Science, and Technology, and Grant from the Foundation for Growth Science, Japan.

The authors have nothing to declare.

Abstract

Context: Mutations of multiple transcription factor genes involved in pituitary development have been identified in a minor portion of patients with combined pituitary hormone deficiency (CPHD). However, copy number aberrations involving such genes have been poorly investigated in patients with CPHD.

Objective: We aimed to report the results of mutation and gene copy number analyses in patients with CPHD.

Subjects and Methods: Seventy-one Japanese patients with CPHD were examined for mutations and gene copy number aberrations affecting *POU1F1, PROP1, HESX1, LHX3, LHX4,* and *SOX3* by PCR-direct sequence and multiplex ligation-dependent probe amplification. When a deletion was indicated, it was further studied by fluorescent in situ hybridization, oligoarray comparative genomic hybridization, and serial sequencing for long PCR products encompassing the deletion junctions.

Results: We identified a *de novo* heterozygous 522,009-bp deletion involving *LHX4* in a patient with CPHD (GH, TSH, PRL, LH, and FSH deficiencies), anterior pituitary hypoplasia, ectopic posterior pituitary, and underdeveloped sella turcica. We also identified five novel heterozygous missense substitutions (p.V201I and p.H387P in *LHX4*; p.T63M and p.A322T in *LHX3*; and p.V53L in *SOX3*) that were assessed as rare variants by sequencing analysis for control subjects and available parents, and by functional studies.

Conclusions: The results imply the rarity of abnormalities affecting the six genes in patients with CPHD and the significance of the gene copy number analysis in such patients.

Abbreviations:

CGH, comparative genomic hybridization; CPHD, combined pituitary hormone deficiency; FISH, fluorescence *in situ* hybridization; MLPA, multiplex ligation-dependent probe amplification.

Pituitary development and function depends on spatially and temporally controlled expression of multiple transcription factor genes such as *POU1F1*, *PROP1*, *HESX1*, *LHX3*, *LHX4*, *SOX3*, and *OTX2* (1–3). Mutations of these genes are usually associated with combined pituitary hormone deficiency (CPHD), although they sometimes lead to isolated GH deficiency (1–3). However, mutations of these genes have been found only in a minor portion of patients with CPHD (2–7). Thus, although multiple genes would remain to be identified in CPHD, a certain fraction of mutations may have been overlooked in these known genes. Indeed, since previous studies have primarily been performed with PCR-direct sequencing for coding exons (4–7), gene copy number aberrations (deletions and duplications) affecting such genes, as well as pathologic mutations in non-coding regions, may remain undetected in patients with CPHD. Indeed, microdeletions of *PROP1* and *LHX3* and microduplications of *SOX3* have been identified in a few of patients with CPHD (8–10).

Thus, we performed sequence and gene copy number analyses for six pituitary transcription factor genes in Japanese patients with CPHD. The results imply the importance of gene copy number analysis in patients with CPHD, while abnormalities of the hitherto known genes still remain very rare in CPHD.

Materials and Methods

Patients

We studied 71 Japanese patients with various types of CPHD (39 males and 32 females; age 1–43 yr). In all the patients, *OTX2* mutations and gene copy number aberrations have been excluded previously (3).

Primers and probes

The primers and probes utilized in this study are summarized in Supplemental Table 1.

Sequence analysis

This study was approved by the Institutional Review Board Committee at National Center for Child Health and Development. After obtaining written informed consent, leukocyte genomic DNA

samples of the 71 patients were amplified by PCR for the all coding exons and their flanking splice sites of *POU1F1, PROP1, HESX1, LHX3, LHX4,* and *SOX3*. Subsequently, the PCR products were subjected to direct sequencing on a CEQ 8000 autosequencer (Beckman Coulter, Fullerton, CA). To confirm a heterozygous substitution, the corresponding PCR products were subcloned with a TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA), and normal and mutant alleles were sequenced separately.

Functional studies

Functional studies were performed for an *LHX4* missense variant. In brief, an expression vector containing the wild-type *LHX4* cDNA was constructed by fusing the human *LHX4* cDNA to the Myc tag in pCMV-Myc (designated as pLHX4-WT). The wild-type *LHX4* cDNA was obtained from a human pituitary cDNA sample (Clontech, Palo, Alto, CA), using primers that were designed to lose the first codon to enable the fusion to the C-terminal sides of the Myc tag. An expression vector containing a variant *LHX4* cDNA (designated as pLHX4-VT) was created by site-directed mutagenesis. The luciferase reporter vector was constructed by inserting the promoter sequences of *POU1F1* with an LHX4 binding site (ATTAATTG) (11) (–541 to +6 bp) into pGL3 basic (pPOU1F1-luc).

Subsequently, transactivation analysis was performed with Dual-Luciferase Reporter Assay System (Promega, Madison, WI). COS1 cells seeded in 12-well dishes ($1.5x10^5$ cells/well) were transiently transfected, using lipofectamine 2000 (Invitrogen), with [1] the empty expression vector (0.6 μg), [2] pLHX4-WT (0.6 μg), [3] pLHX4-VT (0.6 μg), or [4] pLHX4-WT (0.3 μg) plus pLHX4-VT (0.3 μg), together with pPOU1F1-luc (0.6 μg) and pRL-CMV vector (20 ng) used as an internal control for the transfection. Luciferase assays were performed at 48 hours after the transfection with Lumat LB9507 (Berthold, Bad Wildbad, Germany). Transfections were performed in triplicate within a single experiment, and the experiment was repeated three times.

Gene copy number analysis

Muitlplex ligation-dependent probe amplification (MLPA), a recently developed method for relative quantification of single copy sequences (12), was performed as a screening of a possible

gene copy number alteration (deletion and duplication) in all the 71 patients, using a commercially available MLPA probe mix (P236) (MRC-Holland, Amsterdam) for all coding exons of *POU1F1*, *PROP1*, *HESX1*, *LHX3*, and *LHX4*, together with originally designed probes for *SOX3*. The procedure was as described in the manufacturer's instructions. To confirm a deletion, fluorescence *in situ* hybridization (FISH) was performed with a long PCR product obtained using LA taq polymerase (Takara, Ohtsu, Japan). To indicate an extent of a deletion, oligoarray comparative genomic hybridization (CGH) was carried out with 1x244K Human Genome Array (catalog No. G4411B) (Agilent Technologies, CA), according to the manufacturer's protocol. Finally, to characterize a deletion, long PCR was performed with primer pairs flanking the deleted region, and the PCR product was subjected to direct sequencing using serial sequence primers. The deletion size and the junction structure were determined by comparing the obtained sequences with the reference sequences at the NCBI Database (NC_000014.7), and the presence or absence of repeat sequences around the breakpoints was examined with Repeatmasker (http://www.repeatmasker.org).

Results

Mutation analysis

We identified five novel heterozygous missense substitutions, i.e., p.T63M (c.188C>T) and p.A322T (c.964G>A) in *LHX3* (GenBank accession number: NM_178138), p.V201I (c.601G>A) and p.H387P (c.1160A>C) in *LHX4* (NM_033343), and p.V53L (c.157G>C) in *SOX3* (NM_005634). These substitutions were found in different patients. No other mutations or novel substitutions were identified in the six genes examined.

Thus, we examined 100 Japanese control subjects, detecting the *LHX4* p.H387P in 4 subjects and the *SOX3* p.V53L in 3 subjects. Furthermore, sequencing of parental samples was performed for the two *LHX3* substitutions, showing the p.T63M and the p.A322T substitutions in the phenotypically normal mother and father, respectively.

Functional studies

We performed functional studies for the p.V201I substitution in *LHX4*. The p.V201I had a normal transactivation function for the *POU1F1* promoter, with no dominant negative effect

(Supplemental Fig 1).

Deletion analysis

A heterozygous deletion involving *LHX4* was indicated by MLPA and confirmed by FISH (Fig. 1A). Oligoarray CGH delineated an approximately 530-kb deletion, and sequencing of the fusion point showed that the deletion was 522,009 bp in physical size (178,210,910–178,732,918 bp on the NC_000014.7 at the NCBI Database) and was associated with an addition of an 8-bp segment of unknown origin (Fig. 1B). There were no repeat sequences around the deletion breakpoints. This microdeletion was absent from the parents.

Patient with the microdeletion

This Japanese female patient was born at 40 weeks of gestation after an uncomplicated pregnancy and delivery. At birth, her length was 48.0 cm (–0.2 SD), her weight 2.59 kg (–1.0 SD), and her head circumference 33 cm (–0.1 SD). She had transient respiratory distress and hypoglycemia in the early neonatal period. Furthermore, she was found to have hypothyroidism because of prolonged jaundice (Table 1), and was placed on thyroid hormone replacement therapy.

At 1 yr 6 months of age, she was referred to us because of severe short stature. Her height was 64.5 cm (–5.1 SD), and her weight 6.2 kg (–2.8 SD). Endocrine studies indicated GH, TSH, and prolactin deficiencies (Table 1). Her karyotype was 46, XX in all the 50 lymphocytes examined. Recombinant human GH therapy (0.175 mg/kg per week) was started at 1yr 8 months of age, showing a remarkable effect. Brain magnetic resonance imaging at 5 yr of age delineated anterior pituitary hypoplasia with a small cystic lesion, ectopic posterior pituitary, underdeveloped sella turcica (Supplemental Fig. 2). At 11 yr of age, a GnRH test was performed due to lack of pubertal signs, revealing gonadotropin deficiencies. Thus, hormone replacement therapy was started at 13 yr of age. On the last examination at 17 yr old, she measured 148.7 cm (–1.8 SD), weighed 45.6 kg (–0.9 SD), and manifested full pubertal development. She had no developmental retardation.

The non-consanguineous parents and the three brothers were clinically normal. The father was 164 cm (–1.2 SD) tall, and the mother was 155 cm (–0.6 SD) tall.

Discussion

We performed sequence and gene copy number analyses for all coding exons of six previously known genes in 71 patients with CPHD, although non-coding regions were not examined. Consequently, we could identify only a single patient with a heterozygous microdeletion involving *LHX4*. This indicates the rarity of abnormalities affecting the six genes in patients with CPHD and, at the same time, the significance of the gene copy number analysis in such patients. In this regard, since gene copy number aberrations have been found for multiple genes including microdeletions of *PROP1* and *LHX3* and microduplications of *SOX3* (8–10, 13, 14), this implies that a screening of gene copy number aberrations using a simple method such as MLPA should be performed in genetic diagnosis.

Two findings are noteworthy for the microdeletion. First, the microdeletion has removed three additional genes (*CEP350, QSOX1*, and *ACBD6*) (Ensembl, http://www.ensembl.org/). In this context, the pituitary phenotype of the patient with the microdeletion remains within the clinical spectrum of the previously reported patients with heterozygous intragenic loss of function mutations of *LHX4* (Supplemental Table 2) (15–19). In addition, this patient had no discernible extra-pituitary phenotype. Thus, hemizygosity for the three genes would not have a major clinical effect, if any. Second, the deletion break points resided on non-repeat sequences, and the fusion point was associated with an addition of an 8-bp segment of unknown origin. This indicates that the deletion has been produced by nonhomologous end joining, i.e., an aberrant breakage and re-uinion between non-homologous sequences (20).

We also identified five novel heterozygous missense substitutions. However, the p.H387P in *LHX4* and the p.V53L in *SOX3* were identified in control subjects, and the p.T63M and the p.A322T in *LHX3* were found in clinically normal parents. Furthermore, the p.V201I in *LHX4* had a normal transactivation activity for the *POU1F1* promoter. Thus, although the possibility that they might function as a susceptibility factor(s) for the development of CPHD remains tenable, they would not be a disease-causing pathologic mutation. In this regard, p.V201I in *LHX4*, which was absent in 100 control subjects, may have been erroneously regarded as a pathologic mutation, unless functional studies were performed. Such rare variants with an apparently normal function have also been reported previously (3, 18). Thus, while *in vitro* functional data may not precisely reflect *in vivo*

functions, it is recommended to perform functional studies for novel substitutions, especially missense substitutions.

In summary, the results imply the rarity of pathologic abnormalities in the previously known genes in patients with CPHD and the significance of the gene copy number analysis in such patients. Thus, the causes of CPHD remain elusive in most patients, and further studies are required to clarify the underlying factors for the development of CPHD.

ACKNOWLEDGMENTS

We thank the patients and the parents for participating in this study. We also thank Dr. Takizawa and Dr. Morita for participating in medical care of the reported patients.

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