**TABLE 1.** Summary of clinical findings in patients with heterozygous *OTX2* mutations

| | 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.

**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 $\mu$g/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 $\mu$g/m$^2$) | 1.0 | 6.2 | 3.7 | N.E. | 1.3[d] | **6.3** | 1.1 | N.E. | 1.5 | 9.9 |
| TSH ($\mu$U/ml) | TRH (10 $\mu$g/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 $\mu$g/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 ($\mu$g/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 ($\mu$g/liter); LH, 1.0 (IU/liter); FSH, 1.0 (IU/liter); TSH, 1.0 (mIU/liter); prolactin, 1.0 ($\mu$g /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*.

Address all correspondence and requests for reprints to: Dr. T. Ogata, Department of Endocrinology and Metabolism, National Research Institute for Child Health and Development, 2-10-1 Ohkura, Setagaya, Tokyo 157-8535, Japan. E-mail: tomogata@nch.go.jp.

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.

## References

1. Cohen LE, Radovick S 2002 Molecular basis of combined pituitary hormone deficiencies. Endocr Rev 23:431–442
2. Kelberman D, Dattani MT 2007 Hypopituitarism oddities: congenital causes. Horm Res 68(Suppl 5):138–144
3. Vieira TC, Boldarine VT, Abucham J 2007 Molecular analysis of PROP1, PIT1, HESX1, LHX3, and LHX4 shows high frequency of PROP1 mutations in patients with familial forms of combined pituitary hormone deficiency. Arq Bras Endocrinol Metab 51:1097–1103
4. Hever AM, Williamson KA, van Heyningen V 2006 Developmental malformations of the eye: the role of *PAX6*, *SOX2* and *OTX2*. Clin Genet 69:459–470
5. Courtois V, Chatelain G, Han ZY, Le Novère N, Brun G, Lamonerie T 2003 New Otx2 mRNA isoforms expressed in the mouse brain. J Neurochem 84:840–853
6. Ragge NK, Brown AG, Poloschek CM, Lorenz B, Henderson RA, Clarke MP, Russell-Eggitt I, Fielder A, Gerrelli D, Martinez-Barbera JP, Ruddle P, Hurst J, Collin JR, Salt A, Cooper ST, Thompson PJ, Sisodiya SM, Williamson KA, Fitzpatrick DR, van Heyningen V, Hanson IM 2005 Heterozygous mutations of *OTX2* cause severe ocular malformations. Am J Hum Genet 76:1008–1022
7. Wyatt A, Bakrania P, Bunyan DJ, Osborne RJ, Crolla JA, Salt A, Ayuso C, Newbury-Ecob R, Abou-Rayyah Y, Collin JR, Robinson D, Ragge N 2008 Novel heterozygous OTX2 mutations and whole gene deletions in anophthalmia, microphthalmia and coloboma. Hum Mutat 29:E278–E283
8. Dateki S, Fukami M, Sato N, Muroya K, Adachi M, Ogata T 2008 OTX2 mutation in a patient with anophthalmia, short stature, and partial growth hormone deficiency: functional studies using the IRBP, HESX1, and POU1F1 promoters. J Clin Endocrinol Metab 93:3697–3702
9. Diaczok D, Romero C, Zunich J, Marshall I, Radovick S 2008 A novel dominant-negative mutation of OTX2 associated with combined pituitary hormone deficiency. J Clin Endocrinol Metab 93:4351–4359
10. Tajima T, Ohtake A, Hoshino M, Amemiya S, Sasaki N, Ishizu K, Fujieda K 2009 OTX2 loss of function mutation causes anophthalmia and combined pituitary hormone deficiency with a small anterior and ectopic posterior pituitary. J Clin Endocrinol Metab 94:314–319
11. Cartegni L, Chew SL, Krainer AR 2002 Listening to silence and understanding nonsense: exonic mutations that affect splicing. Nat Rev Genet 3:285–298
12. Strachan T, Read AP 2004 Instability of the human genome: mutation and DNA repair. In: Human molecular genetics. 3rd ed. London and New York: Garland Science; 334–337
13. Holbrook JA, Neu-Yilik G, Hentze MW, Kulozik AE 2004 Nonsense-mediated decay approaches the clinic. Nat Genet 36:801–808
14. Schouten JP, McElgunn CJ, Waaijer R, Zwijnenburg D, Diepvens F, Pals G 2002 Relative quantification of 40 nucleic acid sequences by multiplex ligation-dependent probe amplification. Nucleic Acids Res 30:e57
15. Kelley CG, Lavorgna G, Clark ME, Boncinelli E, Mellon PL 2000 The Otx2 homeoprotein regulates expression from the gonadotropin-releasing hormone proximal promoter. Mol Endocrinol 14:1246–1256
16. Matsuo N 1993 Skeletal and sexual maturation in Japanese children. Clin Pediatr Endocrinol 2(Suppl):1–4
17. Chatelain G, Fossat N, Brun G, Lamonerie T 2006 Molecular dissection reveals decreased activity and not dominant-negative effect in human OTX2 mutants. J Mol Med 84:604–615
18. den Dunnen JT, White SJ 2006 MLPA and MAPH: sensitive detection of deletions and duplications. Curr Protoc Hum Genet Chapter 7, Unit 7.14
19. Bennett CP, Betts DR, Seller MJ 1991 Deletion 14q (q22q23) associated with anophthalmia, absent pituitary, and other abnormalities. J Med Genet 28:280–281
20. Elliott J, Maltby EL, Reynolds B 1993 A case of deletion 14(q22.1→q22.3) associated with anophthalmia and pituitary abnormalities. J Med Genet 30:251–252
21. Lemyre E, Lemieux N, Décarie JC, Lambert M 1998 Del(14)(q22.1q23.2) in a patient with anophthalmia and pituitary hypoplasia. Am J Med Genet 77:162–165
22. Nolen LD, Amor D, Haywood A, St Heaps L, Willcock C, Mihelec M, Tam P, Billson F, Grigg J, Peters G, Jamieson RV 2006 Deletion at 14q22–23 indicates a contiguous gene syndrome comprising anophthalmia, pituitary hypoplasia, and ear anomalies. Am J Med Genet A 140:1711–1718

23. Zhu X, Lin CR, Prefontaine GG, Tollkuhn J, Rosenfeld MG 2005 Genetic control of pituitary development and hypopituitarism. Curr Opin Genet Dev 15:332–340
24. Fisher E, Scambler P 1994 Human haploinsufficiency— one for sorrow, two for joy. Nat Genet 7:5–7
25. Parks JS, Felner EI 2007 Hypopituitarism. In: Kliegman RM, Behrman RE, Jenson HB, Stanton BF, eds. Nelson textbook of pediatrics. 18th ed. Philadelphia: Saunders Elsevier; 2293–2299
26. Bouligand J, Ghervan C, Tello JA, Brailly-Tabard S, Salenave S, Chanson P, Lombès M, Millar RP, Guiochon-Mantel A, Young J 2009 Isolated familial hypogonadotropic hypogonadism and a GNRH1 mutation. N Engl J Med 360:2742–2748
27. Suwa S, Tachibana K, Maesaka H, Tanaka T, Yokoya S 1992 Longitudinal standards for height and height velocity for Japanese children from birth to maturity. Clin Pediatr Endocrinol 1:5–13
28. Kuczmarski RJ, Ogden CL, Guo SS, Grummer-Strawn LM, Flegal KM, Mei Z, Wei R, Curtin LR, Roche AF, Johnson CL 2002 2000 CDC growth charts for the United States: methods and development. Vital Health Stat 11 246:1–190
29. Japan Public Health Association 1996 Normal biochemical values in Japanese children (in Japanese). Tokyo: Sanko Press
30. Inada H, Imamura T, Nakajima R 2002 Manual of endocrine examination for children (in Japanese). Osaka: Medical Review

# 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

Sumito Dateki, Maki Fukami, Ayumi Uematsu, Masayuki Kaji, Manami Iso, Makoto Ono, Michiyo Mizota, Susumu Yokoya, Katsuaki Motomura, Eiichi Kinoshita, Hiroyuki Moriuchi, and Tsutomu Ogata

Department of Endocrinology and Metabolism, National Research Institute for Child Health and Development (S.D., M.F., M.I., T.O.), Tokyo 157-8535, Japan; Department of Pediatrics, Nagasaki University Graduate School of Biomedical Sciences (S.D., K.M., E.K., H.M.), Nagasaki 852-8501, Japan; Division of Endocrinology and Metabolism, Shizuoka Children's Hospital (A.U., M.K), Shizuoka 232-8555, Japan; Department of Human Ontogeny and Childhood Development, Tokyo Medical and Dental University (M.O.), Tokyo 113-8519, Japan; Department of Pediatrics, Faculty of Medicine, Kagoshima University, (M.M.), Kagoshima 890-8544, Japan; Department of Medical Subspecialties (S.Y.), National Children's Medical Center, Tokyo 157-8535, Japan.

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.

Address all correspondence and requests for reprints to: Dr. T. Ogata, Department of Endocrinology and Metabolism, National Research Institute for Child Health and Development, 2-10-1 Ohkura, Setagaya, Tokyo 157-8535, Japan. FAX: +81-3-5494-7026, E-mail: tomogata@nch.go.jp

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.

**References**


1. **Cohen LE, Radovick S** 2002 Molecular basis of combined pituitary hormone deficiencies. Endocr Rev 23:431–442
2. **Kelberman D, Rizzoti K, Lovell-Badge R, Robinson IC, Dattani MT** 2009 Genetic regulation of pituitary gland development in human and mouse. Endocr Rev 30:790–829
3. **Dateki S, Kosaka K, Hasegawa K, Tanaka H, Azuma N, Yokoya S, Muroya K, Adachi M, Tajima T, Motomura K, Kinoshita E, Moriuchi H, Sato N, Fukami M, Ogata T** 2009 Heterozygous orthodenticle homeobox 2 mutations are associated with variable pituitary phenotype. J Clin Endocrinol Metab DOI: 10.1210/jc.2009–13
4. **Coya R, Vela A, Pérez de Nanclares G, Rica I, Castaño L, Busturia MA, Martul P; GEDPIT group** 2007 Panhypopituitarism: genetic versus acquired etiological factors. J Pediatr Endocrinol Metab 20:27–36
5. **Vieira TC, Boldarine VT, Abucham J** 2007 Molecular analysis of PROP1, PIT1, HESX1, LHX3, and LHX4 shows high frequency of PROP1 mutations in patients with familial forms of combined pituitary hormone deficiency. Arq Bras Endocrinol Metabol 51:1097–1103
6. **Kim SS, Kim Y, Shin YL, Kim GH, Kim TU, Yoo HW** 2003 Clinical characteristics and molecular analysis of PIT1, PROP1, LHX3, and HESX1 in combined pituitary hormone deficiency patients with abnormal pituitary MR imaging. Horm Res 60:277–283
7. **Rainbow LA, Rees SA, Shaikh MG, Shaw NJ, Cole T, Barrett TG, Kirk JM** 2005 Mutation analysis of POUF-1, PROP-1 and HESX-1 show low frequency of mutations in children with sporadic forms of combined pituitary hormone deficiency and septo-optic dysplasia. Clin Endocrinol (Oxf) 62:163–168
8. **Abrão MG, Leite MV, Carvalho LR, Billerbeck AE, Nishi MY, Barbosa AS, Martin RM, Arnhold IJ, Mendonca BB** 2006 Combined pituitary hormone deficiency (CPHD) due to a complete PROP1 deletion. Clin Endocrinol (Oxf) 65:294–300
9. **Pfaeffle RW, Savage JJ, Hunter CS, Palme C, Ahlmann M, Kumar P, Bellone J, Schoenau E, Korsch E, Brämswig JH, Stobbe HM, Blum WF, Rhodes SJ** 2007 Four novel mutations

of the LHX3 gene cause combined pituitary hormone deficiencies with or without limited neck rotation. J Clin Endocrinol Metab 92:1909–1919

10. **Woods KS, Cundall M, Turton J, Rizotti K, Mehta A, Palmer R, Wong J, Chong WK, Al-Zyoud M, El-Ali M, Otonkoski T, Martinez-Barbera JP, Thomas PQ, Robinson IC, Lovell-Badge R, Woodward KJ, Dattani MT** 2005 Over- and underdosage of SOX3 is associated with infundibular hypoplasia and hypopituitarism. Am J Hum Genet 76:833–849
11. **Machinis K, Amselem S** 2005 Functional relationship between LHX4 and POU1F1 in light of the LHX4 mutation identified in patients with pituitary defects. J Clin Endocrinol Metab 90:5456–5462
12. **Schouten JP, McElgunn CJ, Waaijer R, Zwijnenburg D, Diepvens F, Pals G** 2002 Relative quantification of 40 nucleic acid sequences by multiplex ligation-dependent probe amplification. Nucleic Acids Res 30:e57
13. **Desviat LR, Pérez B, Ugarte M** 2006 Identification of exonic deletions in the PAH gene causing phenylketonuria by MLPA analysis. Clin Chim Acta 373:164–167
14. **Fukami M, Dateki S, Kato F, Hasegawa Y, Mochizuki H, Horikawa R, Ogata T** 2007 Identification and characterization of cryptic SHOX intragenic deletions in three Japanese patients with Léri-Weill dyschondrosteosis. J Hum Genet 53:454–459
15. **Machinis K, Pantel J, Netchine I, Léger J, Camand OJ, Sobrier ML, Dastot-Le Moal F, Duquesnoy P, Abitbol M, Czernichow P, Amselem S** 2001 Syndromic short stature in patients with a germline mutation in the LIM homeobox LHX4. Am J Hum Genet 69:961–968
16. **Tajima T, Hattori T, Nakajima T, Okuhara K, Tsubaki J, Fujieda K** 2007 A novel missense mutation (P366T) of the LHX4 gene causes severe combined pituitary hormone deficiency with pituitary hypoplasia, ectopic posterior lobe and a poorly developed sella turcica. Endocr J 54:637–641
17. **Pfaeffle RW, Hunter CS, Savage JJ, Duran-Prado M, Mullen RD, Neeb ZP, Eiholzer U, Hesse V, Haddad NG, Stobbe HM, Blum WF, Weigel JF, Rhodes SJ** 2008 Three novel missense mutations within the LHX4 gene are associated with variable pituitary hormone deficiencies. J Clin Endocrinol Metab 93:1062–1071

18. **Castinetti F, Saveanu A, Reynaud R, Quentien MH, Buffin A, Brauner R, Kaffel N, Albarel F, Guedj AM, El Kholy M, Amin M, Enjalbert A, Barlier A, Brue T** 2008 A novel dysfunctional LHX4 mutation with high phenotypical variability in patients with hypopituitarism. J Clin Endocrinol Metab 93:2790–2799

19. **Tajima T, Yorifuji T, Ishizu K, Fujieda K** 2009 A novel mutation (V101A) of the LHX4 gene in a Japanese patient with combined pituitary hormone deficiency. Exp Clin Endocrinol Diabetes DOI: 10.1055/s–0029–1225612

20. **Shaw CJ, Lupski JR** 2004 Implications of human genome architecture for rearrangement-based disorders: the genomic basis of disease. Hum Mol Genet 13:R57–R64

**Figure legends**

**Figure 1.** Gene copy number analysis.

A. MLPA and FISH analyses. The black and white boxes on genomic DNA (gDNA) denote the coding regions on exons 1–6 (E1–E6) and the untranslated regions, respectively. The sites examined by MLPA probes (A–F) are indicated by arrows, and the region identified by the 5,305-bp FISH probe is shown by a thick horizontal line. In MLPA analysis, the peaks for the sites A–F are reduced in the patient. The red peaks indicate the internal size markers. In FISH analysis, the red signal is derived from the probe for *LHX4*, and the green signals are derived from chromosome 1 centromere control probe (Cytocell, Cambridge, UK) used as an internal control. The probe for *LHX4* is labeled with digoxigenin and detected by rhodamine anti-digoxigenin, and the control probe is labeled with biotin and detected by avidin conjugated to fluorescein isothiocyanate.

B. Oligoarray CGH analysis and direct sequencing of the deletion junction. The deletion is 522,009 bp in physical size (shaded in gray) and is associated with an addition of an 8-bp segment of unknown origin (highlighted in yellow). The normal sequences flanking the microdeletion are indicated with dashed underlines.

**TABLE 1.** Blood hormone values of the patient with *LHX4* deletion.

| Age at examination | | 1 yr 6 months | | 11 yr | |
|---|---|---|---|---|---|
| | Stimulus (dosage) | Baseline | Peak | Baseline | Peak |
| GH (ng/ml) | GHRH (1 μg/kg) | 0.2 | **1.2** | | |
| | Arginine (0.5 g/kg) | 0.1 | **0.2** | | |
| | L-Dopa (10 mg/kg) | 0.1 | **0.1** | | |
| LH (mIU/ml) | GnRH (100 $\mu g/m^2$) | <0.5 | | 0.3 | **0.8** |
| FSH (mIU/ml) | GnRH (100 $\mu g/m^2$) | 0.5 | | 1.3 | **1.6** |
| TSH (μU/ml) | TRH (10 μg/kg) | 2.3 | **3.9** | | |
| Prolactin (ng/ml) | TRH (10 μg/kg) | **<1.0** | **<1.0** | | |
| ACTH (pg/ml) | | 24.6 | | | |
| Cortisol (μg/dl)[a] | | 17.5 | | | |
| IGF-I (ng/ml) | | **9** | | | |
| Free T4 (ng/dl) | | **0.6**[b] | | | |
| Estradiol (pg/ml) | | | | **<15** | |

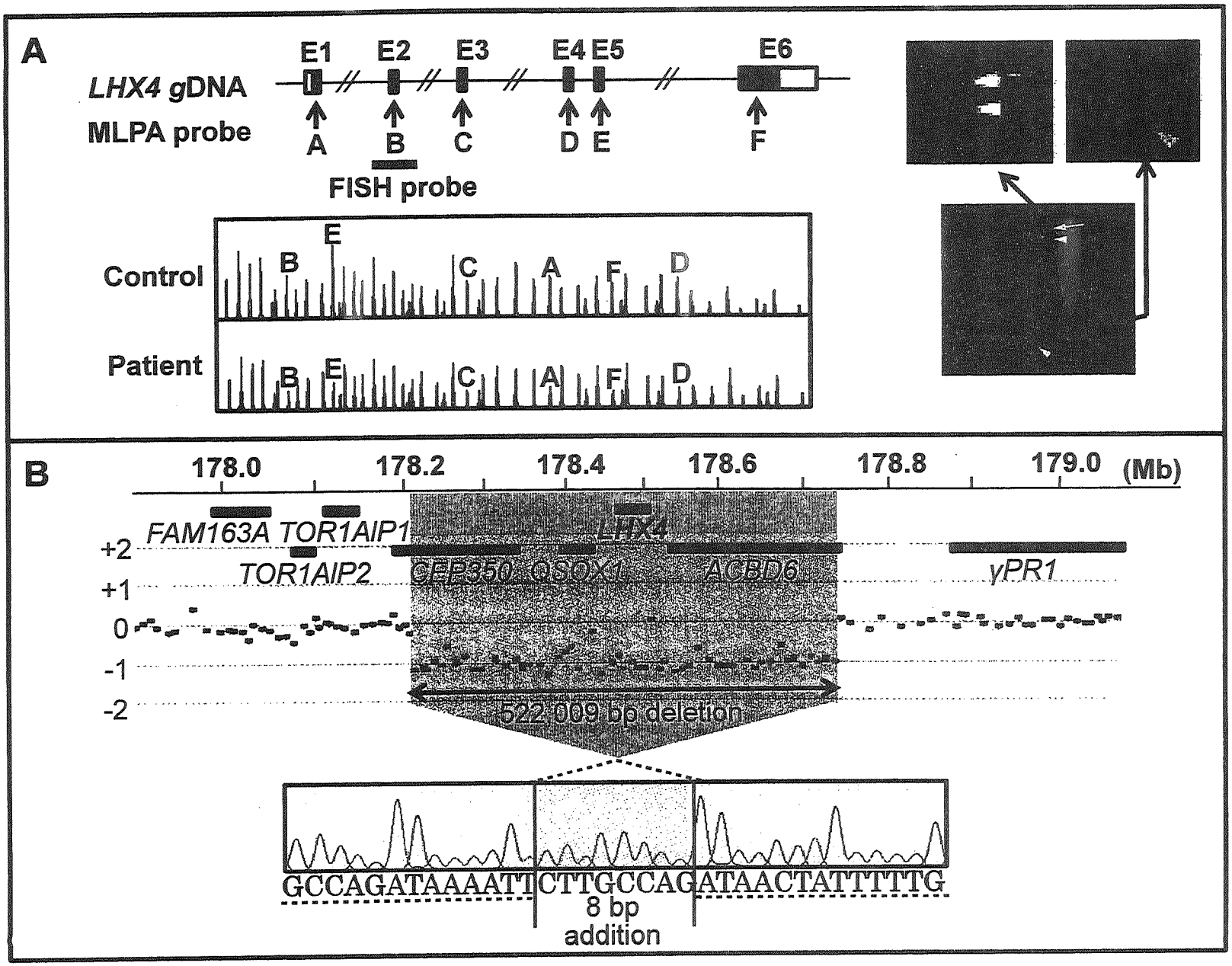
The conversion factor to the SI unit: GH 1.0 (μg/liter), LH 1.0 (IU/liter), FSH 1.0 (IU/liter), TSH 1.0 (mIU/liter), prolactin 1.0 (μg /liter), ACTH 0.22 (pmol/liter), cortisol 27.59 (nmol/liter), IGF-I 0.131 (nmol/liter), free T4 12.87 (pmol/liter), and estradiol 3.671 (pmol/liter).

Hormone values have been evaluated by the age-and sex-matched Japanese reference data; low hormone data are boldfaced.

Blood sampling during the provocation tests: 0, 30, 60, 90, and 120 minutes.

[a] Obtained at 0800h.

[b] Measured at one month of age.


A
E1
E2
E3
E4 E5
E6
LHX4 gDNA
MLPA probe
A
B
C
D E
F
FISH probe
Control
Patient
B
E
C
A
F
D
B
178.0
178.2
178.4
178.6
178.8
179.0
(Mb)
FAM163A
TOR1AIP1
TOR1AIP2
CEP350
QSOX1
LHX4
ACBD6
yPR1
+2
+1
0
-1
-2
522 009 bp deletion
GCCAGATAAAATT
CTTGCCAG
ATAACTATTTTTG
8 bp
addition

# The IG-DMR and the *MEG3*-DMR at Human Chromosome 14q32.2: Hierarchical Interaction and Distinct Functional Properties as Imprinting Control Centers

**Masayo Kagami[1], Maureen J O'Sullivan[2], Andrew J Green[3], Yoshiyuki Watabe[4], Osamu Arisaka[4], Nobuhide Masawa[5], Kentarou Matsuoka[6], Maki Fukami[1], Keiko Matsubara[1], Fumiko Kato[1], Anne C Ferguson-Smith[7], and Tsutomu Ogata[1]***

**1** Department of Endocrinology and Metabolism, National Research Institute for Child Health and Development, Tokyo, Japan, **2** Department of Pathology, Our Lady's Children's Hospital, and School of Medicine, Trinity College, Dublin, Ireland, **3** National Center for Medical Genetics, University College Dublin, Our Lady's Hospital, and School of Medicine and Medical Science, University College, Dublin, Ireland, **4** Department of Pediatrics, Dokkyo University School of Medicine, Tochigi, Japan, **5** Department of Pathology, Dokkyo University School of Medicine, Tochigi, Japan, **6** Department of Pathology, National Center for Child Health and Development, Tokyo, Japan, **7** Department of Physiology, Development and Neuroscience, University of Cambridge, Cambridge, UK.

Running head: Imprinting Control Centers at Human 14q32.2

*Correspondence: tomogata@nch.go.jp.