表 3. POR異常症患者における腎泌尿消化管奇形とatRA濃度

患者			*POR*変異	腎泌尿器	血中atRA
症例	核型	年齢	アミノ酸変化	**消化管奇形**	濃度 (ng/ml)
1	46,XY	2	R457H / R457H	鎖肛	...
2	46,XX	0.4	R457H / R457H	なし	...
3	46,XX	14.1	R457H / R457H	なし	**2.5**
4	46,XX	15	R457H / R457H	尿管逆流	**1.9**
5	46,XX	3	R457H / R457H	なし	**2.5**
6	46,XX	0.1	R457H / R457H	鎖肛	...
7	46,XY	16.8	R457H / Q201X	なし	**2.5**
8	46,XY	14.8	R457H / I444fsX449	なし	**2.4**
9	46,XY	17.5	R457H /delta E1	なし	**1.9**
10	46,XY	2.1	R457H / R48fsX63	腎形成異常	1.5
11	46,XY	0.2	R457H / Q555fsX612	なし	**2.6**
12	46,XX	9	R457H / IVS7+1G>A	なし	1.6
13	46,XX	6.6	R457H / delta E1-2	尿管逆流	**2**
14	46,XX	4.2	R457H / delta E1-2	鎖肛	...
15	46,XY	0.4	R457H / A462-S463insIA	なし	**3.7**
16	46,XY	18	R457H / L612-W620delinsR	尿管逆流	...
17	46,XX	0.8	R457H / E580Q	なし	...
18	46,XX	0.7	R457H / 348delV	鎖肛	**1.8**

atRA: alltrans retinoic acid. **正常値**: 1.0-1.8

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分担研究報告書

診断法の確立と治療指針の作成

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本研究の目的は、適切な社会的性の決定を必要とする新生児期の医学的救急疾患であるのみならず、思春期発来障害、性腺腫瘍易発症性、性同一性障害、不妊症など、生涯にわたり QOL の低下を招く難病である性分化異常症（性分化疾患）の実態把握と、それに基づく治療指針の作成である。本年度では、本年度は、診断法の確立、特に、遺伝子診断法の確立を目指した研究を行った。そして、臨床的アンドロゲン受容体異常症ではSF1変異の関与を、5α還元酵素欠損症では完全酵素欠損においても XY 個体の性自認が生じうることを明確とした。さらに、純粋型XY 性線異形成では世界初となるSOX9周辺の欠失を同定した。これらの所見は、正確な遺伝カウンセリングや長期予後ならびに合併症の調査に大きく貢献すると考えられる

A. 研究目的

本研究の目的は、性分化疾患（性分化異常症）における診断法の確立と治療指針の作成である。これにより、性分化疾患における医療の均てん化に貢献することである。なお、本研究班の対象疾患は多岐に亘り、多くの性分化疾患を伴う先天奇形症候群も含む。

本研究の必要性は、本症が、適切な社会的性の決定を必要とする新生児期の医学的救急疾患であるのみならず、思春期発来障害、性腺腫瘍易発症性、性同一性障害、不妊症など、生涯にわたりQOLの低下を招く難病であり、2006年の性分化異常症国際会議で、本症における初期対応から長期にわたる管理戦略の構築と共に、遺伝子解析による原因疾患診断や発症率・長期予後解析の重要性が確認された点にある。本研究班は、性分化異常症に従事する研究者、小児内分泌科医師、小児泌尿器科医師から構成され、関連学会と連携して活動する。

この分担研究では、診断法の確立と治療指針（手引き）の作成を主眼とする。そして、治療指針については、昨年度に作成した「性分化疾患初期対応の手引」（日本小児科学会雑誌掲載、本研究班および日本小児内分泌学会ホームページ掲載）に引き続き、研究分担者である堀川玲子が「性分化疾患小児期対応の手引」の作成に従事したため、われわれは、診断法の確立、特に、遺伝子診断法の確立を目指した研究を行った。具体的には、最も頻度が高く、かつ、臨床診断と遺伝子診断に不一致がみられる「臨床的アンドロゲン受容体異常症」、外性器の状態に関わらず遺伝的男児を社会的男児として養育すべきと考えられるようになった「5α還元酵素欠損症」、そして、最も原因不明例が多い「XY 性線異形成」を重点的に解析した。

B. 研究方法

- 臨床的アンドロゲン受容体異常症：完全型 7 名例、不完全型約 70 例において、責任遺伝子 AR 解析を行った。さらに、変異陰性例において、SF1 や MAMLD1 などの候補遺伝子解析、ならびに SF1 胎児ライディッヒ細胞特異的エンハンサー解析を行った。
- 5α還元酵素欠損症：家系例を対象として、責任遺伝子 SRD5A2 解析を行った。このデータを社会的性の決定に活用した。
- 純粋型 XY 性線異形成：性分化疾患以外に症状のない約 30 例の患者を対象として、既知責任遺伝子 SF1 や SOX9、ならびに候補遺伝子 LHX9, DMRT1 の解析を行った。さらに、SOX9 周辺の欠失解析や全ゲノムの CGH 解析を行った。

（倫理面への配慮）

ヒト検体を使用する際には、文部科学・厚生労働・経済産業省「ヒトゲノム・遺伝子解析研究に関する倫理指針」を遵守して研究を実施する。ヒト検体を採取する際には、試料等提供者のプライバシーの保護、検体提供の任意性、研究参加者の利益および不利益、提供を受けた検体の取り扱い方、得られる研究成果の医学的貢献度等について、試料等提供者ないしはその保護者に充分説明したうえで、文書により同意を得る。また、試料等の提供を求める際に、説明文書を用いて分かりやすく、かつ充分に説明し、必要に応じて遺伝カウンセリングを行う。なお、本研究に関連する内容は、全て倫理委員会の承認済である。

C. 研究結果

- 臨床的アンドロゲン受容体異常症：完全型の患者では、約 70%において AR 遺伝子変異が同定されたが、不完全型の患者では 10%以下の患者においてのみ AR 遺伝子変異が見いだされた。さらに、臨床的に不完全型アンドロゲン受容体異常症と診断された患者の中で、SF1 のヘテロレフレームシフト変異が同定された（図1）。この家系では、発端者（3女：遺伝的男性で、社会的女性）が臨床的アンドロゲン受容体異常症を呈し、同じ変異を有する二女（遺伝的男性で、社会的女性）が臨床的に聖染色体異形成と診断されていた。さらに、母親にも同じ変異があり、この母親は、生理不順などの軽度卵巣機能不全を示していた。また、長女（遺伝的い女性で、社会的女性）も同じ変異を有することから、将来の本人の卵巣機能不全や子供の性分化疾患発症が危惧された。

また、SF1 胎児ライディッヒ細胞特異的エンハンサーの異常が理論的に臨床的アンドロゲン受容体異常症を生じると考えられることから解析したが、明らかな異常は認められなかった。

- 5α 還元酵素欠損症：第 1 子（遺伝的男性で、社会的女性）が軽度外陰部男性化のために検査され、臨床的に 5α 還元酵素欠損症と診断された家系を解析した。この児は、生後間もなくから男児的な行動をとり、様々な心理的検査から性自認の障害が疑われていた。その後、第 4 子（遺伝的男性）が同様の外陰部異常症を呈したため、SRD5A2 解析を行った結果、父親がこの遺伝子欠失の保因者であり、母親がこの遺伝子欠失のナンセンス変異の保因者であり、第 1 子と第 4 子（および遺伝的女児である第 2 子）が SRD5A2 の複合ヘテロ変異患者であり、理論的に 5α 還元酵素の完全欠損症と判明した（図 2）。その後、両親を交えた話し合いで、第 4 子を社会的男児として養育することとなった。
- 純粋型 XY 性線異形成：既知責任遺伝子 SF1 変異が同定されたことの他に、SOX9 周辺の欠失が同定された。これは世界初の成果であり、SOX9 遺伝子の性線特異的エンハンサーの同定に繋がるものである。

D. 考察

- 臨床的アンドロゲン受容体異常症：本研究のデータは、責任遺伝子である AR 変異が、完全型患者の約 75%、不完全型患者の約 20%程度にしか同定されないという過去のデータと一致するものである。おそらく、軽度の性線異形成などが、臨床的アンドロゲン受容体異常症に含められていると推測されている。これに関し、精巣異形成を生じることが判明している SF1 遺伝子変異が1家系において同定されたことは注目される。これは、SF1 変異が臨床的アンドロゲン受容体異常症を生じうることを明確にするものである。さらに、この家系の変異陽性者の遺伝子型－表現型解析から、SF1 変異を持つ XY 個体の臨床像が同一家系内でも大きく異なりうること、SF1 変異が卵巣機能障害を生じうることを示す点で情報価値の高いものと考えらえる。
- 5α 還元酵素欠損症：近年、5α 還元酵素欠損症を持つ遺伝的男性患者の性自認の解析から、外陰部が女児に近いために女児として養育された患者が、生後（特に思春期から）自身を男性と認識することが判明してきている。そのため、最近では、外陰部の形態に関わらず、5α 還元酵素欠損症を持つ遺伝的男性患者は社会的に男児として養育すべきであるという意見が提唱されている。本症例は、これに一致するものであり、さらに、5α 還元酵素の完全欠損を有する患者においても、性自認の揺らぎが生じることを世界で初めて明確とするものである。
- 純粋型 XY 性線異形成：現在、純粋型 XY 性線異形成患者では、約 10%程度の頻度で SRY 変異が認められるものの、これ以外の原因はほとんど判明していない。さらに、近年、SOX9 の関与が次第に明確になりつつあるが、現在まで、骨症状のない純粋型 XY 性線異形成患者において SOX9 の SOX9 周辺の欠失の存在を明らかとするもので、SOX9 遺伝子の性線特異的エンハンサーの同定に繋がると期待される。

E. 結論

本年度は、診断法の確立、特に、遺伝子診断法の確立を目指した研究を行った。そして、臨床的アンドロゲン受容体異常症では SF1 変異の関与を、5α 還元酵素欠損症では完全酵素欠損においても XY 個体の性自認が生じうることを明確とした。さらに、純粋型 XY 性線異形成では SOX9 周辺の欠失を同定したこれらの所見は、正確な遺伝カウンセリングや長期予後ならびに合併症の調査に大きく貢献すると考えられる。

F. 健康危険情報

特記すべきことなし。

G. 研究発表

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2. 学会発表

省略

H. 知的財産権の出願・登録状況

（予定を含む。）

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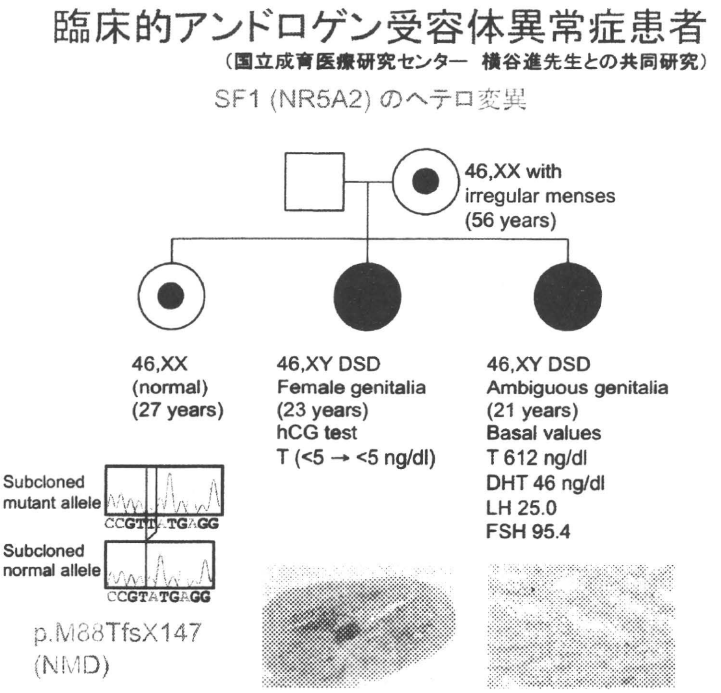

図 1. 臨床的アンドロゲン受容体異常症患者（第 3 子）とその家系における SF-1 変異の同定と遺伝子型－表現型解析

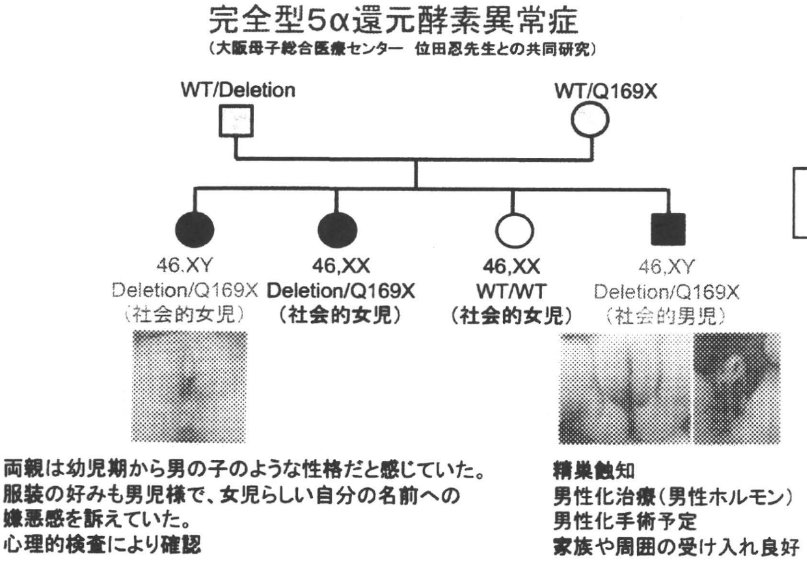

図 2. 5α 還元酵素欠損症家系

研究成果の刊行一覧表

別紙4

雑誌

発表者氏名	論文タイトル名	発表誌名	巻名	ページ	出版年
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研究成果の刊行物・別刷り

Heterozygous Orthodenticle Homeobox 2 Mutations Are Associated with Variable Pituitary Phenotype

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Context: Although recent studies have suggested a positive role of *OTX2* in pituitary as well as ocular development and function, detailed pituitary phenotypes in *OTX2* mutations and *OTX2* target genes for pituitary function other than *HESX1* and *POU1F1* remain to be determined.

Objective: We aimed to examine such unresolved issues.

Subjects: We studied 94 Japanese patients with various ocular or pituitary abnormalities.

Results: We identified heterozygous p.K74fsX103 in case 1, p.A72fsX86 in case 2, p.G188X in two unrelated cases (3 and 4), and a 2,860,561-bp microdeletion involving *OTX2* in case 5. Clinical studies revealed isolated GH deficiency in cases 1 and 5; combined pituitary hormone deficiency in case 3; abnormal pituitary structures in cases 1, 3, and 5; and apparently normal pituitary function in cases 2 and 4, together with ocular anomalies in cases 1–5. The wild-type Orthodenticle homeobox 2 (OTX2) protein transactivated the *GNRH1* promoter as well as the *HESX1*, *POU1F1*, and *IRBP* (interstitial retinoid-binding protein) promoters, whereas the p.K74fsX103-OTX2 and p.A72fsX86-OTX2 proteins had no transactivation functions and the p.G188X-OTX2 protein had reduced (~50%) transactivation functions for the four promoters, with no dominant-negative effect. cDNA screening identified positive *OTX2* expression in the hypothalamus.

Conclusions: 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*. (***J Clin Endocrinol Metab* 95: 756–764, 2010**)

Pituitary development and function depends on the spatially and temporally controlled expression of multiple transcription factor genes such as *POU1F1*, *HESX1*, *LHX3*, *LHX4*, *PROP1*, and *SOX3* (1, 2). Whereas mutations of some genes (*e.g. POU1F1*) result in a relatively characteristic pattern of pituitary hormone deficiency, those of other genes (*e.g. HESX1*) are associated with a wide range of pituitary phenotype including combined pi-

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Abbreviations: CGH, Comparative genomic hybridization; CPHD, combined pituitary hormone deficiency; EPP, ectopic posterior pituitary; FISH, fluorescence *in situ* hybridization; HD, homeodomain; IGHD, isolated GH deficiency; IRBP, interstitial retinoid-binding protein; MLPA, multiplex ligation-dependent probe amplification; NMD, nonsense mediated mRNA decay; OTX2, orthodenticle homeobox 2; PH, pituitary hypoplasia; SOD, septooptic dysplasia; TD, transactivation domain.

tuitary hormone deficiency (CPHD), isolated GH deficiency (IGHD), and apparently normal phenotype. However, because mutations of these genes account for a relatively minor portion of patients with congenital hypopituitarism (2, 3), multiple genes would remain to be identified in congenital hypopituitarism.

Orthodenticle homeobox 2 (*OTX2*) is a transcription factor gene primarily involved in ocular development (4). It encodes a paired type homeodomain (HD) and a transactivation domain (TD) and produces two functionally similar splice variants, isoform-a (GenBank accession no. NM_21728.2) and isoform-b (NM_172337.1) with and without eight amino acids because of alternative splice acceptor sites at the boundary of intron 3 and exon 4 (5). To date, at least 10 pathological heterozygous *OTX2* mutations have been identified in patients with ocular malformations such as anophthalmia and/or microphthalmia (6, 7). Ocular phenotype is highly variable, ranging from anophthalmia to nearly normal eye development, even in patients from the same family. Furthermore, most patients also exhibit brain anomaly, seizure, and/or developmental delay.

Recent studies have indicated that *OTX2* is also involved in pituitary development and function. Dateki *et al.* (8) showed that *OTX2* is expressed in the pituitary and has a transactivation function for the promoters of *POU1F1* and *HESX1* as well as the promoter of *IRBP* (interstitial retinoid-binding protein) involved in ocular function and that a frameshift *OTX2* mutation identified in a patient with bilateral anophthalmia and partial IGHD barely retained the transactivation activities. Subsequently a missense *OTX2* mutation with a dominant-negative effect and a frameshift *OTX2* mutation with loss-of-function effect were identified in CPHD patients with and without ocular malformation (9, 10).

However, detailed pituitary phenotypes in *OTX2* mutation-positive patients as well as other possible *OTX2* target genes for pituitary development and function remain to be determined. Here we report five new patients with *OTX2* mutations and summarize clinical findings in *OTX2* mutation-positive patients. We also show that *OTX2* is expressed in the hypothalamus and has a transactivation function for the promoter of *GNRH1*.

Patients and Methods

Patients

We studied 94 Japanese patients consisting of: 1) 16 patients with ocular anomalies and pituitary dysfunctions accompanied by short stature (< −2 SD) (six with anophthalmia and/or microphthalmia and CPHD, five with anophthalmia and/or microphthalmia and IGHD, three with septooptic dysplasia (SOD) and CPHD, and two with SOD and IGHD) (group 1); 2) 12 patients with ocular anomalies whose pituitary functions were not investigated (one with bilateral microphthalmia and short stature, one with bilateral optic nerve hypoplasia and short stature, and 10 with anophthalmia and/or microphthalmia and normal stature) (group 2); and 3) 66 patients with pituitary dysfunctions but without ocular anomalies (five with IGHD and 61 patients with CPHD) (group 3). No demonstrable mutation was identified for *HESX1* in patients with SOD, *GH1* and *HESX1* in patients with IGHD, and *POU1F*, *HESX1*, *LHX3*, *LHX4*, *PROP1*, and *SOX3* in patients with various types of CPHD (2). All the patients had normal karyotype.

Primers and probes

The primers and probes used in this study are shown in Supplemental Table 1, published as supplemental data on The Endocrine Society's Journals Online web site at http://jcem.endojournals.org.

Sequence analysis of OTX2

This study was approved by the Institutional Review Board Committee at National Center for Child Health and Development. After obtaining written informed consent, the coding exons 3-5 and their flanking splice sites were PCR amplified using leukocyte genomic DNA samples of all 94 patients and were subjected to direct sequencing on a CEQ 8000 autosequencer (Beckman Coulter, Fullerton, CA). To confirm a heterozygous mutation, the corresponding PCR products were subcloned with TOPO TA cloning kit (Invitrogen, Carlsbad, CA), and normal and mutant alleles were sequenced separately.

Prediction of the occurrence of aberrant splicing and nonsense mediated mRNA decay (NMD)

To examine whether identified mutations could cause aberrant splicing by creating or disrupting exonic splicing enhancers and/or splice sites (11, 12), we performed *in silico* analyses with the ESE finder release 3.0 (http://rulai.cshl.edu/cgi-bin/tools/ESE3/esefinder.cgi) for the prediction of exonic splice enhancers and with the program at the Berkeley Drosophila Genome Project (http://www.fruitfly.org/seq_tools/splice.html) for the prediction of splice sites. We also analyzed whether identified mutations could be subject to NMD on the basis of the previous report (12, 13).

Deletion analysis

Multiplex ligation-dependent probe amplification (MLPA) was performed for *OTX2* intragenic mutation-negative patients as a screening of a possible microdeletion affecting *OTX2*. This procedure was performed according to the manufacturer's instructions (14), using probes designed specifically for *OTX2* exon 4 together with a commercially available MLPA probe mix (P236) (MRC-Holland, Amsterdam, The Netherlands) used as internal controls. To confirm a microdeletion, fluorescence *in situ* hybridization (FISH) was performed with a long PCR product for *OTX2* (a 6096 bp segment from intron 2 to exon 5) together with an RP11-566I2 BAC probe (14q11.2; Invitrogen, Carlsbad, CA) used as an internal control. The probe for *OTX2* was labeled with digoxigenin and detected by rhodamine anti-digoxigenin, and the control probe was labeled with biotin and

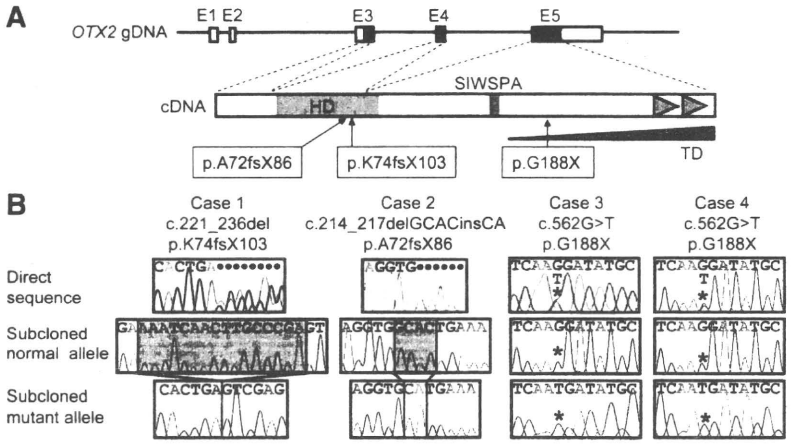

FIG. 1. Sequence analysis in cases 1–4. A, The structure of *OTX2* (the isoform-b) and the position of the mutations identified. The *black* and *white boxes* on genomic DNA (gDNA) denote the coding regions on exons 1-5 (E1-E5) and the untranslated regions, respectively. *OTX2* encodes the HD (a *blue 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

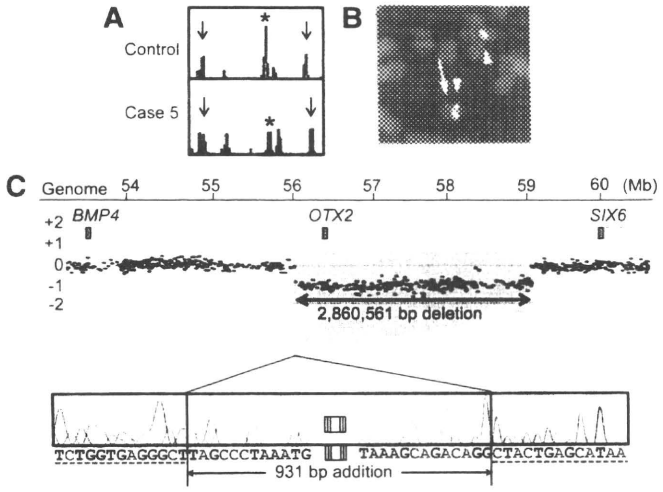

FIG. 2. Deletion analysis in case 5. A, MLPA analysis. The *red asterisk* indicates peaks for the *OTX2* exon 4, and the *black arrows* indicate control peaks. The *red peaks* indicate the internal size markers. Deletion of the MLPA probe binding site is indicated by the reduced peak height. B, FISH 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*.

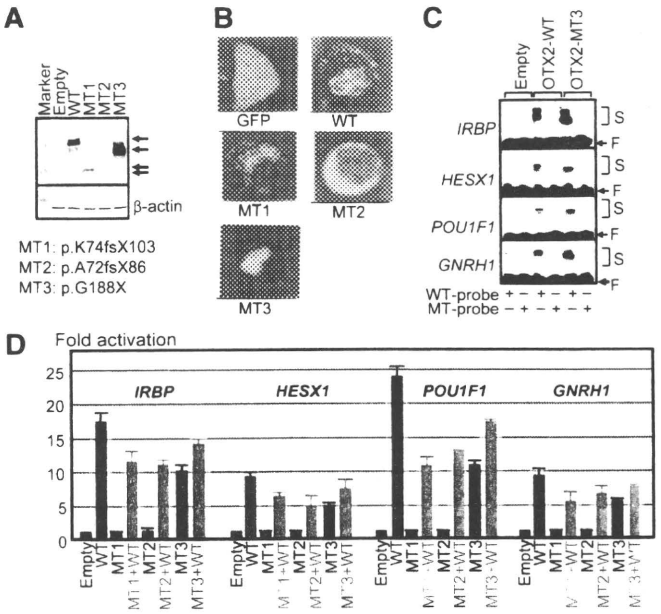

FIG. 3. Functional studies. A, Western blot analysis. Both WT and MT1-MT3 OTX2 proteins are detected with different molecular masses (*arrows*). WT, Wild type; MT1, p.K74fsX103; MT2, p.A72fsX86; and MT3, p.G188X. B, Subcellular localization analysis. Whereas green fluorescent protein (GFP) alone is diffusely 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

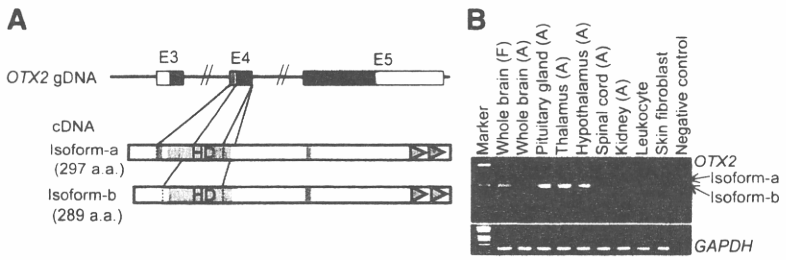

FIG. 4. PCR-based human cDNA library screening for *OTX2* (35 cycles). A, Schematic representation of the *OTX2* isoform-a (NM_21728.2) and isoform-b (NM_172337.1). Because of the two alternative splice acceptor sites at the boundary between intron 3 and exon 4, isoform-a carries eight amino acids (shown 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-

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 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.

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Novel Insights from Clinical Practice

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Hypothalamic Dysfunction in a Female with Isolated Hypogonadotropic Hypogonadism and Compound Heterozygous *TACR3* Mutations and Clinical Manifestation in Her Heterozygous Mother

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Established Facts

- *TAC3* and *TACR3* have recently been shown to be causative genes for an autosomal recessive form of isolated hypogonadotropic hypogonadism (IHH).

Novel Insights

- Hypothalamic dysfunction may be the primary cause for IHH in patients with biallelic *TACR3* mutations.
- Clinical phenotype may be exhibited by females with heterozygous *TACR3* mutations.
- *TAC3* and *TACR3* mutations remain rare in patients with IHH.

Key Words
Heterozygous manifestation · Hypogonadotropic hypogonadism · Hypothalamus · *TACR3* mutation

Abstract
Background/Aims: *TAC3* and *TACR3* have recently been shown to be causative genes for an autosomal recessive form of isolated hypogonadotropic hypogonadism (IHH). Here, we report a Japanese female with IHH and compound heterozygous *TACR3* mutations and her heterozygous parents, and discuss the primary lesion for IHH and clinical findings. ***Case Report:*** This female was identified through mutation analysis of *TAC3* and *TACR3* in 57 patients with IHH. At 24 years of age, an initial standard GnRH test showed poor gonadotropin response (LH <0.2–0.6 IU/l), whereas the second GnRH test performed after GnRH priming (100 μg i.m. for 5

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