

Table 3. Clinical Manifestations in NS Patients with *RAF1*, *PTPN11*, *SOS1*, and *KRAS* Mutations

	<i>RAF1</i> ^a (%)	<i>PTPN11</i> ^b (%)	<i>SOS1</i> ^c (%)	<i>KRAS</i> ^d (%)
Total patients	52	172	73	18
Perinatal abnormality				
Polyhydramnios	12/34 (35)	ND	9/16 (56)	2
Fetal macrosomia	11/31 (35)	ND	9/15 (60)	ND
Growth and development				
Failure to thrive in infancy	13/15 (87)	35/56 (63)	ND	3/3 (100)
Mental retardation	25/45 (56)	71/164 (43)	12/67 (18) ^e	16/17 (94) ^f
Outcome				
Died	6/28 (21)	ND	ND	ND
Craniofacial characteristics				
Relative macrocephaly	32/38 (84)	ND	9/21 (43) ^e	9/11 (82)
Hypertelorism	34/36 (94)	15/28 (54) ^f	5/6 (83)	12/12 (100)
Downslanting palpebral fissures	29/37 (78)	19/28 (68)	20/22 (91)	9/12 (75)
Ptosis	28/37 (76)	18/29 (62)	19/24 (79)	10/15 (67)
Epicanthal folds	24/35 (69)	15/28 (54)	ND	2/9 (22) ^f
Low set ears	32/36 (89)	56/64 (88)	20/22 (91)	7/10 (70)
Skeletal characteristics				
Short stature	41/50 (82)	97/172 (56) ^e	22/58 (38) ^e	12/17 (71)
Short neck	35/46 (76)	15/29 (52) ^e	17/22 (77)	9/10 (90)
Webbing of neck	38/46 (83)	36/122 (30) ^e	3/6 (50)	7/14 (50) ^e
Cardiac defects				
Hypertrophic cardiomyopathy	37/51 (73)	10/135 (7) ^e	7/73 (10) ^e	3/18 (17) ^e
Septal defect	22/52 (42)	41/170 (24) ^e	17/73 (23) ^e	5/18 (28)
Atrial septal defect	16/51 (31)			4/18 (22)
Ventricular septal defect	6/52 (12)			1/18 (6)
Pulmonic stenosis	11/50 (22)	125/171 (73) ^f	53/73 (73) ^f	7/18 (39)
Patent ductus arteriosus	2/20 (10)	ND	ND	1/18 (6)
Mitral valve anomaly	13/49 (27)	ND	ND	3/18 (17)
Arrhythmia	14/25 (56)	ND	ND	ND
Skeletal/extremity deformity				
Cubitus valgus	9/31 (29)	14/61 (23)	1/6 (17)	2/2 (100)
Pectus deformity	25/44 (57)	108/171 (63)	38/56 (68)	13/16 (81)
Skin/hair anomaly				
Curly hair	14/41 (34)	ND	15/22 (68) ^f	1/12 (8)
Hyperelastic skin	12/33 (36)	ND	1/6 (17)	3/12 (25)
Café au lait spots	3/34 (9)	ND	1/6 (17)	1/9 (11)
Lentiginosities	3/35 (9)	ND	ND	ND
Naevus	12/37 (32)	ND	ND	ND
Genitalia				
Cryptorchidism	13/23 (57)	75/138 (54)	22/39 (56)	4/11 (36)
Blood test abnormality				
Coagulation defects	3/15 (20)	46/90 (51)	14/66 (21)	2/9 (22)

ND, not described.

^a[Ko et al., 2008; Pandit et al., 2007; Razzaque et al., 2007]; and this study.

^b[Jongmans et al., 2005; Musante et al., 2003; Tartaglia et al., 2002; Zenker et al., 2004].

^c[Ferrero et al., 2008; Ko et al., 2008; Narumi et al., 2008; Roberts et al., 2007; Tartaglia et al., 2007; Zenker et al., 2007a].

^d[Carta et al., 2006; Ko et al., 2008; Lo et al., 2008; Schubbert et al., 2006; Zenker et al., 2007b].

^eThe frequency of the manifestation in patients with the gene was significantly lower compared with that observed in *RAF1*-positive patients ($P < 0.05$ by Fisher's exact test).

^fThe frequency of the manifestation in patients with the gene was significantly higher compared with that observed in *RAF1*-positive patients ($P < 0.05$ by Fisher's exact test).

approximately 30% of NS and related disorders remain unknown. Presently unknown genetic causes for mutation-negative NS and related disorders remain to be identified in molecules in future studies.

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Original article

A familial case of LEOPARD syndrome associated with a high-functioning autism spectrum disorder

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Abstract

A connection between LEOPARD syndrome (a rare autosomal dominant disorder) and autism spectrum disorders (ASDs) may exist. Of four related individuals (father and three sons) with LEOPARD syndrome, all patients exhibited clinical symptoms consistent with ASDs. Findings included aggressive behavior and impairment of social interaction, communication, and range of interests. The coexistence of LEOPARD syndrome and ASDs in the related individuals may be an incidental familial event or indicative that ASDs is associated with LEOPARD syndrome. There have been no other independent reports of the association of LEOPARD syndrome and ASDs. Molecular and biochemical mechanisms that may suggest a connection between LEOPARD syndrome and ASDs are discussed.

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Keywords: LEOPARD syndrome, Noonan syndrome; Autism spectrum disorders (ASDs); RAS/MAPK signal transduction pathway

1. Introduction

LEOPARD syndrome (OMIM#151100) is a rare autosomal dominant disorder characterized by Lentigines, Electrocardiogram abnormalities, Ocular hypertelorism, Pulmonic valvular stenosis, Abnormalities of genitalia, Retardation of growth, and Deafness. This syndrome is caused by germline missense mutations in the *PTPN11* gene that encodes Src homology 2 domain-containing tyrosine phosphatase 2 (Shp2): non-receptor protein-tyrosine phosphatase comprising two N-terminal SH2 domains, a catalytic domain, and a C

terminus with tyrosylphosphorylation sites and a proline-rich stretch. The mutations induce catalytically impaired Shp2 by a “dominant negative effect” [1–2].

In the more common Noonan syndrome, approximately 50% of patients have *PTPN11* mutations scattered over the entire Shp2, including the catalytic domain. The mutations resulting in the Noonan phenotype are the “gain-of-function” mutations, and they exhibit substantially increased catalytic ability. Although LEOPARD syndrome and Noonan syndrome are caused by *PTPN11* mutations resulting in opposite effects, they share many common clinical features, including physical dysmorphic findings and intellectual disability [1].

The term “autism spectrum disorders (ASDs)” was first used by Lorna Wing [3] and then widely used as a category comprised of autistic disorder, Asperger’s

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disorder, and other related conditions [4]. These conditions are very common neurobehavioral disorders that are characterized by impairments in three behavioral domains, including social interaction, language/communication/imaginative play, and a range of interests and activities [3–5].

At least ten genes have been reported to be associated with ASDs [6]. Except for Rett syndrome, the other pervasive developmental disorder (PDD) subtypes including autistic disorder, Asperger's disorder, disintegrative disorder, and PDD Not Otherwise Specified (PDDNOS) are not tightly linked to any particular gene mutations. Several common genetic syndromes are known to be associated with ASDs. Autism is frequent in patients with tuberous sclerosis (TSC) [7], with neurofibromatosis type 1 [8,9] and with Fragile X syndrome [10]. Studies of psychological profiles of adults with Noonan syndrome did not suggest a specific behavioral phenotype, but difficulties with social competence and emotional perceptions were noted [11]. A case of Noonan syndrome who was also diagnosed with autism was reported [12]. The present study of neuropsychiatric evaluation in a familial case of LEOPARD syndrome indicates all patients satisfied the criteria of ASDs. An association of LEOPARD syndrome and ASDs has not been reported previously. The familial case presented in this report may suggest such an association.

2. Patients and methods

After obtaining written informed consent, fifteen coding exons in *PTPN11* were sequenced in each patient following the methods described somewhere else [13].

Diagnostic and Statistical Manual of Mental Disorders, Fourth Edition (DSM-IV-TR) [5] and The high-functioning Autism Spectrum Screening Questionnaire (ASSQ) [14] were used in neuropsychiatric evaluation of the subjects.

Patient 1 is a 20-year-old male who was born as the second child to a non-consanguineous Japanese couple. His early developmental milestones were reportedly unremarkable. He was clinically diagnosed with LEOPARD syndrome at age 7 years based on findings that included lentigines, multiple café-au-lait spots, electrocardiogram (ECG) abnormalities, ventricular septal defect, ocular hypertelorism, short stature, and unilateral renal hypoplasia. *PTPN11* mutation analysis revealed a heterozygous mutation of 1403C > T (T468 M). The patient was diagnosed as having Asperger's disorder based on ASSQ and DSM-IV-TR, at age 12 years. His intelligence quotient (IQ) by the Wechsler Intelligence Scale for Children-third edition (WISC-III) was 85 (verbal: 77, performance 98). His ASSQ score by mother's rating was 41. He met the DSM-IV-TR diagnostic criteria of Asperger's disorder with all subcategories in the category of Qualitative impairment in social interaction

(Category 1), three subcategories (1,2, and 4) in the category of Restricted repetitive and stereotyped patterns of behavior, interests and activities (Category 2), and the rest of the four categories (Table 1).

Patient 2 is a 15-year-old younger brother of Patient 1. His early infantile developmental milestones were unremarkable. He was diagnosed with growth retardation at age 2½ years. At age 12 years his clinical findings of a few café-au-lait spots, ocular hypertelorism, and undescended testes led us to obtain *PTPN11* mutation analysis, which showed the same heterozygous mutation of 1403C > T. At age 9 years, a diagnosis of Asperger's disorder was made based on ASSQ and DSM-IV-TR. His full-scale IQ by WISC-III at age 9 years was 99 (verbal 104, performance 92). His ASSQ score by parental rating was 32 at age 15 years. He also met the Asperger's disorder diagnostic criteria with all subcategories of Category 1, three of Category 2 (1, 2, and 4), and the rest of the categories (Table 1).

Patient 3 is the 22-year-old eldest brother of Patients 1 and 2. His developmental milestones were normal, although his ritualistic behavior and difficulties in relating to peers were noted in his childhood. He had a surgical repair of bilateral undescended testes and inguinal hernia. He was diagnosed with Wolf-Parkinson-White syndrome at age nine years. He has ocular hypertelorism and short stature. The same *PTPN11* heterozygous mutation found in the two younger siblings was identified in this patient. He attends college, and was diagnosed as having PDDNOS, because he also had impaired development of reciprocal social interaction associated with communication skills, repetitive routine, and ritualistic behavior. His ASSQ score was 7 at age 22 years (Table 1).

Patient 4 is a 55-year-old male who is the father of the siblings. He has prominent lentigines, bilateral mild hearing loss, cardiac anomalies, ECG abnormalities, short stature, and apparent ocular hypertelorism. His early developmental milestones are not well known. He has been noted to have obsession with a specific topic, repetitive routine and rituals, and clumsy movements. At age 50 years, his social skills and aggressive behavior were noted to be deteriorating, and consequently he was suspected of having Asperger's disorder based on DSM-IV-TR. He met the diagnostic criteria of Asperger's disorder with Category 1 (1 and 3), Category 2 (1 and 2), and the rest of the four categories. His ASSQ score was 20 at age 55 years by his wife's evaluation. He has the same heterozygous *PTPN11* mutation (Table 1).

3. Discussion

The presented familial case of LEOPARD syndrome included individuals (patients 1, 2, and 4) diagnosed with or suspected of having Asperger's disorder, and

Table 1
Summary of clinical findings and *PTPN11* mutation.

	Pt. 1 Male	Pt. 2 Male	Pt. 3 Male	Pt. 4 Male
Age	20 y	15 y	22 y	55 y
<i>Physical findings</i>				
Skin: café-au-lait spots	multiple	a few	a few	a few
Lentiginosities	+++	+++	–	+++
Cardiac defects	VSD	No	No	No
EKG abnormalities	+	No	WPW	No
Ocular hypertelorism	+	+	+	+
Pulmonary stenosis	No	No	No	No
Abnormal genitalia	No	Und. Testes*	Und. Testes*	No
Renal anomalies	R-hypoplasia	No	No	No
Retardation of growth	Yes	+	+	No
Deafness	No	No	No	Yes
<i>Miscellaneous:</i>				
Rocker bottom feet	Yes	Yes	Yes	No
Macrocephaly	Yes	Yes	Yes	No
<i>PTPN11</i> mutation	T468 M	T468 M	T468 M	T468 M
<i>Neuropsychological</i>				
Diagnosis	AD**	AD**	PDDNOS***	AD**
ASSQ score ⁽¹⁾ (age)	41 (12 y)	32 (15 y)	7 (22 y)	20 (50 y)
WISC-III ⁽²⁾ (age)	85 (12 y)	99 (9 y)	n/a	n/a
-Verbal/performance	77/98	104/92	n/a	n/a

* Und. Testes, undescended testes.

** AD, Asperger's disorder.

*** PDDNOS, Pervasive developmental disorder not otherwise specified.

(1) ASSQ score, Autism Spectrum Screening Questionnaire Score. The cutoff score of 3 predicts 91% of the true positive rate of Autistic spectrum disorders.

(2) WISC-III, Wechsler Intelligence Scale for Children-third edition.

patient 3 was diagnosed as having PDDNOS, which may lead to the diagnosis of ASD. ASDs were first introduced by Lorna Wing, who suggested that Asperger's disorder is a type of ASD and described in detail its various manifestations in speech, nonverbal communication, social interaction, motor coordination, motor clumsiness, and idiosyncratic interests [3]. Patient 3 did not have enough clinical symptoms to meet the diagnostic criteria for Asperger's disorder; however, he had some symptoms suggestive of ASD in his childhood that led to a diagnosis of PDDNOS.

The ASSQ is a 27-item checklist for completion by lay informants when assessing characteristic symptoms of Asperger's disorder and high-functioning autism in children and adolescents with normal intelligence or mild mental retardation. The ASSQ allows for rating on a 3-point scale (0, 1, or 2; 0 indicating normality, 1 some abnormality, and 2 definite abnormality). The range of possible scores is 0–54. The mean ASSQ parent scores in the Asperger's disorder validation sample were 25.1 (SD, 7.3) [14]. The cutoff score of 13 is 91% of the true positive rate of ASDs. The ASSQ score was established as a screening tool primarily for children between 6 and 17 years of age by parents and/or teachers. The delayed evaluation of patient 3 may account for the difference in diagnosis between this patient and his siblings.

ASDs are known to be associated with particular genetic disorders such as fragile X syndrome [10,15,

16], tuberous sclerosis (TSC) [7], and neurofibromatosis type 1 [8,9]. Fifty percent of children with TSC have behavioral problems in the form of ASDs. Gene mutations in either *TSC1* or *TSC2* influence neural precursors, resulting in abnormal cell differentiation and dysregulated control of cell size. These cells migrate to the cortex to generate an abnormal collection of inappropriately positioned neurons, causing widespread cortical disorganization and structural abnormalities [7]. Mutations in *PTPN11* causing LEOPARD syndrome induce catalytically impaired Shp2. In situ hybridization detected Shp2 expression in the neural ectoderm and nervous system in mouse embryos, suggesting an involvement of Shp2 in neural development. Shp2 is a critical signaling molecule in the coordinated regulation of progenitor cell proliferation and neuronal/astroglial cell differentiation. The studies with mutant mouse strains with Shp2 selectively deleted in neural precursor cells showed a dramatic phenotype of growth retardation, early postnatal lethality, and multiple defects in proliferation and cell fate specification in neural stem/progenitor cells [17]. The product of the *TSC2* gene tuberlin is known to up-regulate the B-RAF/MEK/MAPK signal transduction pathway. B-RAF is required for neuronal differentiation, suggesting another possible link between B-RAF signaling and the clinical manifestations of TSC including ASDs [18]. Disturbed neuronal cell differentiation and development due to mutations in

the TSC genes and the *PTPN11* gene are likely to contribute to the development of ASDs in patients with these syndromes.

NF-I is well known to be associated with ASDs. The prevalence of autism in patients with NF-I was reported to be 4% [9]. The well-known function of the NF-I protein is to act as a RAS-GTPase-activating protein known to be involved in the regulation of the RAS-mitogen-activated protein kinase (MAPK) pathway. Mutations in the NF-I gene are thought to result in activation of the RAS/MAPK signal transduction pathway [2]. Clinical overlap between LEOPARD syndrome and NF-I is also well known [19].

Approximately 50% of patients with Noonan syndrome are due to missense *PTPN11* mutations [20]. *PTPN11* encodes SHP2, a protein tyrosine phosphatase, that is involved in the activation of the RAS/MAPK cascade [2]. Noonan syndrome is caused by “gain of function” *PTPN11* mutations [1,2], and the SHP2 mutants due to the *PTPN11* mutations causing Noonan syndrome cause prolonged activation of the RAS/MAPK pathway [2]. Schubbert et al. [21] reported that germline KRAS mutations cause Noonan syndrome through the hyperactive RAS/MAPK pathway.

Herauld et al. [22] reported a positive association of the HRAS gene and autism. The psychological profiles of adults and children with Noonan syndrome have been studied, and deficiencies in social and emotional recognition and expression have been identified in adults, while low verbal IQ, clumsiness, and impairment of developmental coordination have been reported in children [23].

To date, there have been no reports to suggest an association of LEOPARD syndrome and ASDs. Our observations in this familial case may suggest at least some degree of association between LEOPARD syndrome and ASD phenotypes possibly through the RAS/MAPK signal transduction pathway. Further studies with more patients with LEOPARD syndrome are needed to establish the association and to investigate the genetic contributing factors causing ASDs, leading to the prevention and earlier detection of ASDs and better management of patients with these disorders.

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Implications of Prenatal Diagnosis of the Fetus With Both Interstitial Deletion and a Small Marker Ring Originating From Chromosome 5

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We describe a patient with 47,XY,del(5)(p11p13), +mar observed in prenatal screening. We performed analyses including G-banding, multi-color fluorescent in situ hybridization (mFISH) for fetal chromosome detection. After birth array-based comparative genomic hybridization (aCGH), bacterial artificial chromosome (BAC)-FISH was carried out to define the chromosomal changes precisely. The mFISH revealed that a ring chromosome that had originated from chromosome 5. The aCGH showed that this fetus had a terminal duplication, an interstitial deletion, and a pericentromeric duplication of the short arm of chromosome 5. This complex alteration resulted in partial trisomy 5p15.33–p15.31, partial monosomy 5p14.3–p13.2, and partial trisomy 5p12–p11. To clarify these alterations, we performed BAC-FISH using BAC clones related to deleted and duplicated regions, and found that a derivative (der) chromosome 5 showed the presence of hybridization signals from the duplicated region at 5p15.33 and the loss of hybridization signals from the deleted region at 5p14.2. In addition, FISH analysis confirmed the origin of the marker chromosome. Hybridization signals from the second intervening sequence at 5p13.1, between the deleted region and the pericentric duplicated region, were present on the marker ring chromosome. © 2010 Wiley-Liss, Inc.

Key words: BAC-FISH; microarray analysis; prenatal diagnosis; ring chromosome

INTRODUCTION

When genetic abnormalities are observed during prenatal screening, deletions, and supernumerary ring chromosomes are often seen separately [Gardner and Sutherland, 1996; Ryan et al., 1997; Slavotinek and Kingston, 1997]. Most cases with deletion of autosomes, even that of a tiny segment, are accompanied by clinical symptoms, including mental and developmental retardation; on

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the other hand, there have been examples of cases with deletion of autosomes without any abnormal features [Gardner and Sutherland, 1996; Daniel and Malafej, 2003; Liehr et al., 2004].

Here, we describe a rare case with both interstitial deletion and a small ring originating from the same chromosome 5 detected prenatally and characterized by molecular cytogenetics. We emphasize the usefulness of molecular cytogenetics involving array-based comparative genomic hybridization (aCGH) and bacterial artificial chromosome (BAC)-fluorescent in situ hybridization BAC-FISH in providing precise information in cases of complex structural abnormality.

CLINICAL REPORT

Amniocentesis requested for advanced maternal age was performed in gestational week 16 on a 42-year-old woman with two

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normal children. Fetal chromosomes were analyzed by GTG banding and multi-color fluorescent in situ hybridization (mFISH) using cultured amniocytes. After cytogenetic analyses, she was informed that one chromosome 5 with interstitial deletion and a small marker ring chromosome were detected in all the cells. Then, chromosomal analysis of the parents was performed on peripheral blood and showed normal karyotypes. It was difficult to offer additional molecular analyses within a limited term for pregnancy interruption. Ultrasonographic examination at 19 weeks of gestation did not detect any specific abnormality in the fetus. Despite possible unfavorable prognosis informed in genetic counseling, she and her spouse decided against termination of the pregnancy.

The pregnancy was uneventful and she delivered a phenotypically normal boy at 39 weeks of gestation. Apgar score was 8/8 and there were no particular clinical features. Body length, weight, and head circumference were within the normal range: 48 cm, 2,916 g, and 33 cm, respectively. After birth, we received informed consent to examine aCGH and BAC-FISH for further confirmation of the diagnosis.

Developmental, physical, and neurological examinations were normal and he appropriately reached his milestones. At 1 year and 6 months, his developmental quotient (DQ) was 110 (Fig. 1); echocardiography and brain imaging were also normal.

MOLECULAR CYTOGENETIC STUDIES

Chromosome and FISH Analyses

Cultured amniocytes were analyzed using G-banding with 540 bands per haploid number. G-banded chromosomes demonstrated

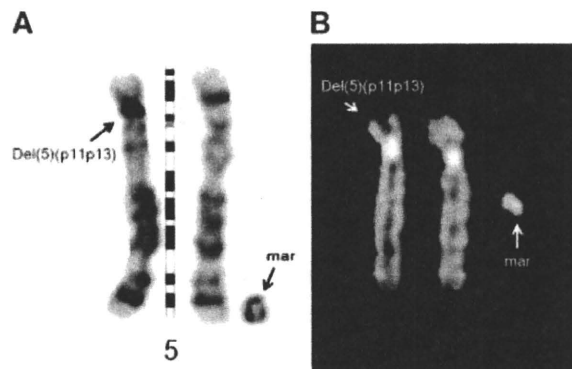


FIG. 2. Partial karyogram of chromosome 5, del(5p), and supernumerary ring by G-banding (A) and mFISH (B).

that all cells had an interstitial deletion of chromosome 5 (5p11 → p13), and a small marker ring chromosome (Fig. 2A). The origin of this marker chromosome was unclear by G-banding. Therefore, the initial karyotyping was 47,XY,del(5)(p11p13), +mar. The mFISH revealed that the marker ring originated from chromosome 5, the same as the deleted chromosome (Fig. 2B). The ring chromosome seemed to have a centromere because this marker was detected in all cells. Chromosome analysis of the parents showed no abnormalities, indicating that these structural abnormalities in the fetus were de novo.

Oligonucleotide aCGH

For detection of gain and loss of chromosome segments, oligonucleotide-based microarray analysis was performed on reserved cultured amniocytes using a 105K-feature whole-genome microarray (Signature Chip Oligo Solution[®], made for Signature Genomic Laboratories by Aligent Technologies Inc., Santa Clara, CA) [Ballif et al., 2008]. Microarray analysis of 1543 loci using on oligonucleotide array detected a complex abnormality in the DNA obtained from cultured amniocytes. Based on microarray analysis, this fetus had two duplications and a deletion of the short arm of chromosome 5. This abnormality was first characterized by a single copy gain of 380 oligonucleotide probes from the terminal end of the short arm of 5p, at 5p15.33p15.31. The extent of this duplication has been estimated to be approximately 6.1 Mb. The second alteration was characterized by a single copy loss of 347 oligonucleotide probes from 5p14.3p13.2. The extent of this interstitial deletion is estimated to be approximately 15.3 Mb. The third alteration was characterized by a single copy gain of 147 oligonucleotide probes from the pericentric region at 5p12p11. The extent of this duplication has been estimated to be approximately 3.4 Mb. Thus, this complex alteration resulted in partial trisomy 5p15.33–p15.31, partial monosomy 5p14.3–p13.2, and partial trisomy 5p12–p11. In conclusion, the result of microarray was arr5p15.33–p15.31(131,945–6,267,160)x3, 5p14.3–p13.2(21,438,495–36,736,934)x1, 5p12–p11(42,529,343–45,908,725)x3 (Fig. 3).



FIG. 1. Propositus at age 8 months (left picture) and 1 year 6 months (right picture). Note the phenotypically normal boy.

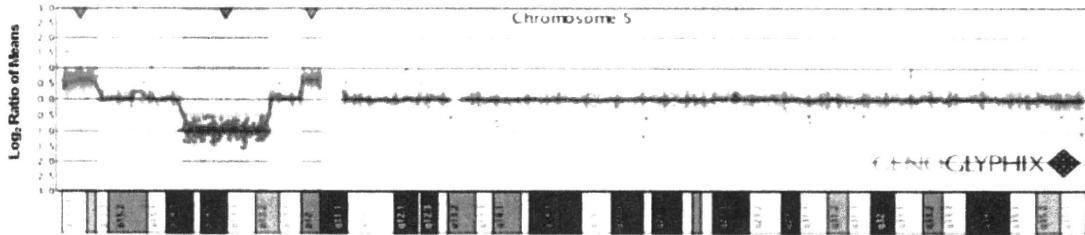


FIG. 3. Microarray plot showing, from left to right, a single copy gain of 380 oligonucleotide probes at 5p15.33p15.31, approximately 6.1 Mb in size; a single copy loss of 347 oligonucleotide probes from 5p14.3p13.2, approximately 15.3 Mb; and single copy gain of 147 oligonucleotide probes at 5p12p11, approximately 3.4 Mb in size. Probes are ordered on the x-axis according to physical mapping positions, with the distal p-arm on the left and the distal q-arm on the right.

BAC-FISH

For confirmation of the array results, FISH analyses were performed with BAC clones from duplicated and deleted regions as previously described [Shaffer et al., 1994; Traylor et al., 2009]. For this study, we used cord blood obtained at delivery.

FISH using a BAC clone from the 5p14.2 deleted region (RP11-701M20) and the 5p15.33 duplicated region (RP11-1006P13) identified an abnormal deleted (del) chromosome 5 that showed the loss of hybridization signals from the deleted region at 5p14.2 (Fig. 4A) and the presence of hybridization signals from the duplicated region at 5p15.33 (Fig. 4C). Interphase FISH

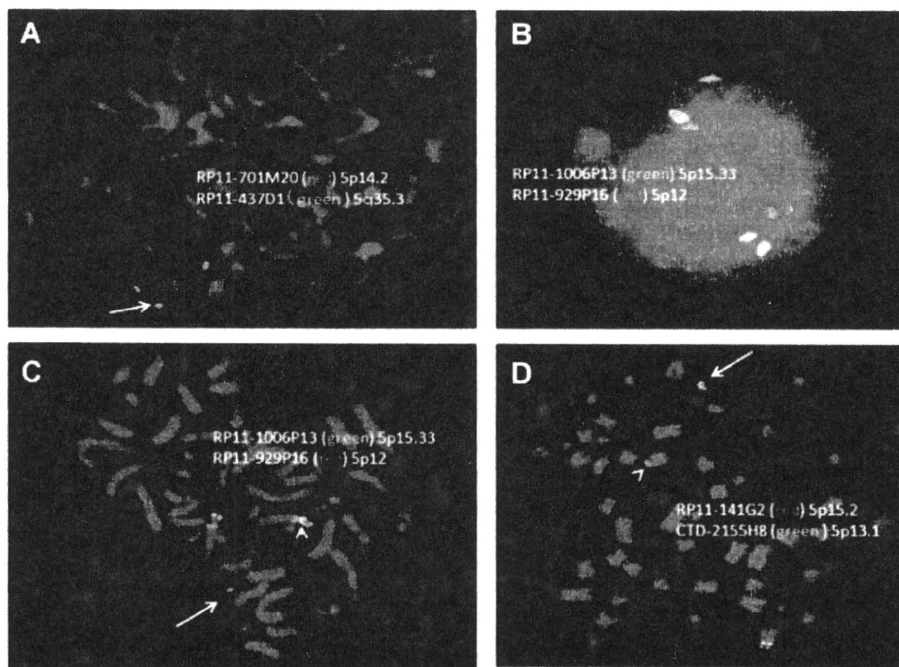


FIG. 4. FISH characterizations of a complex rearrangement on the short arm of chromosome 5. **A:** FISH showing a deletion of 5p14.2, BAC clone RP11-701M20 from 5p14.2 is labeled in red, and RP11-437D1 from 5q35.3 is labeled in green as a control. The presence of one red signal indicates deletion of 5p14.2 on one homologue (arrow). **B,C:** FISH with probes from the two regions is shown to be present in three copies by aCGH. BAC clone RP11-1006P13 from 5p15.33 is labeled in green, and RP11-929P16 from 5p12 is labeled in red. Interphase FISH (B) confirmed the presence of three copies of both regions. Metaphase FISH (C) shows a red signal but not a green signal on a small, supernumerary ring chromosome (arrow), indicating the presence of the 5p12 material on the supernumerary chromosome. Dotted green signals from 5p15.33 were present on the normal chromosome 5 homologs, but terminal duplicated signals were observed on one chromosome 5 (arrowhead). **D:** FISH with probes from the intervening regions shown to be present in two copies by aCGH. BAC clone RP11-141G2 from 5p15.2 is labeled in red, and CTD-2155H8 from 5p13.1 is labeled in green. The supernumerary ring chromosome (arrow) shows a green signal and therefore the presence of material from 5p13.1, while one chromosome 5 homolog (arrowhead) shows a deletion for this region. The probe from 5p15.2 shows a normal hybridization pattern.

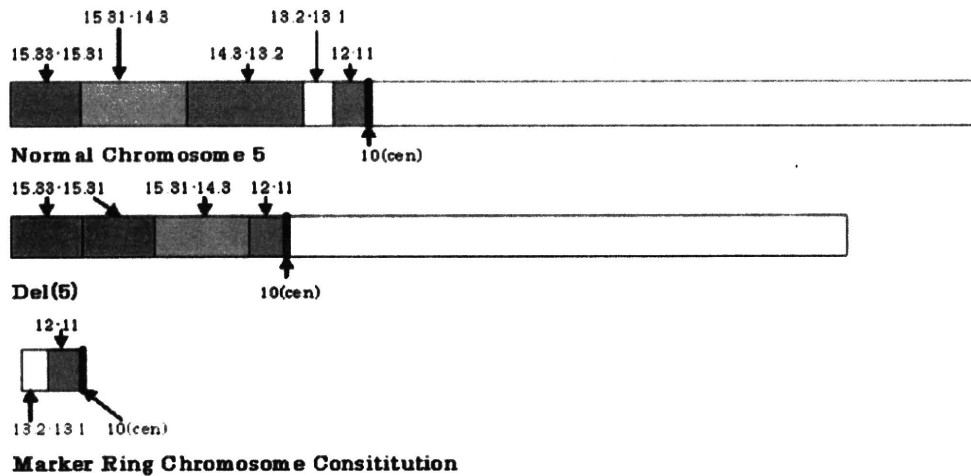


FIG. 5. Molecular background information on the deleted chromosome 5 and marker ring chromosome.

(Fig. 4B) clarified the presence of three copies of 5p15.33 and 5p12 regions. This del(5) also showed hybridization signals in an experiment using BAC clones from the first normal intervening sequence, between the terminal duplication and the deleted region, at 5p15.2 (RP11-141G2; Fig. 4D). Additional FISH analysis using a BAC clone from the 5p12 duplicated region (RP11-929P16) confirmed the origin of the marker ring chromosome (Fig. 4C). Hybridization signals from the second intervening normal sequence at 5p13.1 (CTD-2155H8), between the deleted region and the pericentric duplicated region, were also present on the marker ring chromosome, but not on the del(5), indicating that the deletion on that chromosome extends from 5p14.3 through 5p13.1 (Fig. 4D).

In conclusion, this baby had two abnormal derivative chromosomes. The first der(5) had an abnormal short arm with a duplication of 5p15.31 → 5p15.33, and a deletion of 5p13.1 → p14.3. The second der(5), the marker ring chromosome, was comprised of material from 5p10 → p13.2 (Fig. 5). The final karyotype of the baby is: 47,XY,ish der(5)(pter → p15.31::pter → p14.3::p11 → qter)(RP11-1006P13++,RP11-141G2+,RP11-701M20-,CTD-2155H8-),+der(5):(13.2 → p10):(CTD-2155H8+,RP11-929P16+).

DISCUSSION

Partial deletion of 5p is often seen in prenatal diagnoses and newborn analyses [Mainardi et al., 2001; Weiss et al., 2003]. In autosomes other than chromosome 5, deletions involving various chromosomes have also been reported in the literature [Gardner and Sutherland, 1996; Ryan et al., 1997; Slavotinek and Kingston, 1997]. Partial deletion of autosomes is generally accompanied by mild-to-severe clinical symptoms, including mental and developmental retardation in babies, although there have been exceptional cases where no clinical symptoms are observed [Callen et al., 1993; Overhauser et al., 1994; Knight et al., 1995]. Supernumerary marker chromosomes including small rings are also seen frequently in prenatal diagnoses [Michalski et al., 1993; Brøndum-Nielsen and

Mikkelsen, 1995; Karaman et al., 2006]. Among babies with such small markers, some cases have no clinical features, but others showed mild-to-severe abnormalities after birth [Callen et al., 1993; Overhauser et al., 1994; Knight et al., 1995; Gardner and Sutherland, 1996; Daniel and Malafiej, 2003; Liehr et al., 2004; Bernardini et al., 2007]. Thus, in genetic counseling, it is important to offer chromosomal information from prenatal diagnoses and to provide as much detail as possible, including the origin and inheritance.

The present case had a deletion and a supernumerary marker ring chromosome. To our knowledge, this is the first report of detection by prenatal screening of both a deletion and a marker ring. In the literature, there are some mosaic cases of clones with a deletion and an additional ring separately [Gutiérrez-Angulo et al., 2002; Gereltzul et al., 2008; Kara et al., 2008], but such cases are extremely rare. In newborn infants, only one other case has been reported [Schuffenhauer et al., 1996] with a deletion and a ring of chromosome 5; this baby showed a mosaicism of 46,XY,del(5)/47,XY,del(5),+dic(5), with macrocephaly, asymmetric square skull, minor facial anomalies, omphalocele, inguinal hernias, hypospadias, and club feet. The break points of the deletion shared cen and p13 with those of the dicentric ring chromosome; this case had partial duplication of 5p (p13 → cen), and the mechanisms of del(5) and dic(5) were relatively straightforward. In the present case, on the other hand, the mechanisms of del(5) and marker ring [r(5)] were extremely complex. Microarray analysis revealed a terminal duplication, an interstitial deletion, and a pericentromeric duplication of the short arm of chromosome 5. Through this analysis, a total of six break points of the short arm of chromosome 5 (p15.33, p15.31, p14.3, p13.2, p12, and p11) were related to the formation of the structural abnormality with the duplication and the deletion, and the marker ring. According to the results of the G-band analysis of this case, we determined the break points of del(5) to be p11 and p13. However, assuming the microarray data are correct, the composition of r(5) becomes complicated, and explanation of the underlying mechanisms becomes difficult. To facilitate understanding of the exact composition of del(5) and r(5),

we performed FISH analysis using BAC clones from the duplicated 5p15.33, 5p12 regions and deleted 5p14.2 region. The short arm of the del(5) revealed a duplication of 5p15.31 → 5p15.33 and a deletion of 5p13.1 → p14.3. The r(5) was comprised of material from 5p10 → p13.2. Although supernumerary ring chromosome formation is difficult to determine, we speculate that this case have resulted from “centromere misdivision” along with a break in either the p or q arm, forming a small ring chromosome [Baldwin et al., 2008].

In summary, this complex 5p abnormality was characterized by a terminal duplication of 5p15.33p15.31 of approximately 6.4 Mb, an interstitial deletion 15p14.3p13.2 of approximately 15.3 Mb and an interstitial duplication of 5p12p11 of approximately 3.4 Mb. The 5p terminal duplication contained at least 21 genes including ADAMTS16, AHRR, and C5orf38. The 5p14.3p13.2 deletion lacked at least 22 genes including CDH12, PRDM9, CDH10, and DH9. The 5p12p11 duplication contained at least 11 genes including GHR, SEPP1, C5orf39, and ZNF11131.

When the child was examined at 1 year and 6 months, we could not find any developmental abnormality, either physical or mental. Because of his age he will need to be followed to confirm normal intellectual development. In order to provide accurate and useful genetic counseling in similar cases in the future, the accumulation of further reports with complicated chromosome abnormalities would be beneficial.

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SHORT COMMUNICATION

Androgenetic/biparental mosaicism in a girl with Beckwith–Wiedemann syndrome-like and upd(14)pat-like phenotypes

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This report describes androgenetic/biparental mosaicism in a 4-year-old Japanese girl with Beckwith–Wiedemann syndrome (BWS)-like and paternal uniparental disomy 14 (upd(14)pat)-like phenotypes. We performed methylation analysis for 18 differentially methylated regions on various chromosomes, genome-wide microsatellite analysis for a total of 90 loci and expression analysis of *SNRPN* in leukocytes. Consequently, she was found to have an androgenetic 46,XX cell lineage and a normal 46,XX cell lineage, with the frequency of the androgenetic cells being roughly calculated as 91% in leukocytes, 70% in tongue tissues and 79% in tonsil tissues. It is likely that, after a normal fertilization between an ovum and a sperm, the paternally derived pronucleus alone, but not the maternally derived pronucleus, underwent a mitotic division, resulting both in the generation of the androgenetic cell lineage by endoreplication of one blastomere containing a paternally derived pronucleus and in the formation of the normal cell lineage by union of paternally and maternally derived pronuclei. It appears that the extent of overall (epi)genetic aberrations exceeded the threshold level for the development of BWS-like and upd(14)pat-like phenotypes, but not for the occurrence of other imprinting disorders or recessive Mendelian disorders.

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Keywords: androgenesis; Beckwith–Wiedemann syndrome; mosaicism; upd(14)pat

INTRODUCTION

A pure androgenetic human with paternal uniparental disomy for all chromosomes is incompatible with life because of genomic imprinting.^{1,2} However, a human with an androgenetic cell lineage could be viable in the presence of a normal cell lineage. Indeed, an androgenetic cell lineage has been identified in six liveborn individuals with variable phenotypes.^{3–7} All the androgenetic cell lineages have a 46,XX karyotype, and this is consistent with the lethality of an androgenetic 46,YY cell lineage.

Here, we report on a girl with androgenetic/biparental mosaicism, and discuss the underlying factors for the phenotypic development.

CASE REPORT

This patient was conceived naturally to non-consanguineous and healthy parents. At 24 weeks gestation, the mother was referred to us because of threatened premature delivery. Ultrasound studies showed Beckwith–Wiedemann syndrome (BWS)-like features,⁸ such as macroglossia, organomegaly and umbilical hernia, together with

polyhydramnios and placentomegaly. The mother repeatedly received amnioreduction and tocolysis.

She was delivered by an emergency cesarean section because of preterm rupture of membranes at 34 weeks of gestation. Her birth weight was 3730 g (+4.8 s.d. for gestational age), and her length 45.6 cm (+0.7 s.d.). The placenta weighed 1040 g (+7.3 s.d.).⁹ She was admitted to a neonatal intensive care unit due to asphyxia. Physical examination confirmed a BWS-like phenotype. Notably, chest roentgenograms delineated mild bell-shaped thorax characteristic of paternal uniparental disomy 14 (upd(14)pat),¹⁰ although coat hanger appearance of the ribs indicative of upd(14)pat was absent (Supplementary Figure 1). She was placed on mechanical ventilation for 2 months, and received tracheostomy, glossectomy and tonsillectomy in her infancy, due to upper airway obstruction. She also had several clinical features occasionally reported in BWS⁸ (Supplementary Table 1). Her karyotype was 46,XX in all the 50 lymphocytes analyzed. On the last examination at 4 years of age, she showed postnatal growth failure and severe developmental retardation.

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MOLECULAR STUDIES

This study was approved by the Institutional Review Board Committee at the National Center for Child health and Development, and performed after obtaining informed consent.

Methylation analysis

We first performed bisulfite sequencing for the *H19*-DMR (differentially methylated region) and KvDMR1 as a screening of BWS^{11,12} and that for the IG-DMR and the *MEG3*-DMR as a screening of upd(14)pat,¹⁰ using leukocyte genomic DNA. Paternally derived clones were predominantly identified for the four DMRs examined (Figure 1a). We next performed combined bisulfite restriction analysis for multiple DMRs, as reported previously.¹³ All the autosomal DMRs exhibited markedly skewed methylation patterns consistent with predominance of paternally inherited clones, whereas the *XIST*-DMR on the X chromosome showed a normal methylation pattern (Figure 1a).

Genome-wide microsatellite analysis

Microsatellite analysis was performed for 90 loci with high heterozygosities in the Japanese population.¹⁴ Major peaks consistent with paternal uniparental isodisomy and minor peaks of maternal origin were identified for at least one locus on each chromosome, with the minor peaks of maternal origin being more obvious in tongue and

tongil tissues than in leukocytes (Figure 1b and Supplementary Table 2). There were no loci with three or four peaks indicative of chimerism. The frequency of the androgenetic cells was calculated as 91% in leukocytes, 70% in tongue cells and 79% in tonsil cells, although the estimation apparently was a rough one (for details, see Supplementary Methods).

Expression analysis

We examined *SNRPN* expression, because *SNRPN* showed strong expression in leukocytes (for details, see Supplementary Data). *SNRPN* expression was almost doubled in the leukocytes of this patient (Figure 1c).

DISCUSSION

These results suggest that this patient had an androgenetic 46,XX cell lineage and a normal 46,XX cell lineage. In this regard, both the androgenetic and the biparental cell lineages appear to have derived from a single sperm and a single ovum, because a single haploid genome of paternal origin and that of maternal origin were identified in this patient by genome-wide microsatellite analysis. Thus, it is likely that after a normal fertilization between an ovum and a sperm, the paternally derived pronucleus alone, but not the maternally derived pronucleus, underwent a mitotic division, resulting both in the generation of the androgenetic cell lineage by endoreplication of

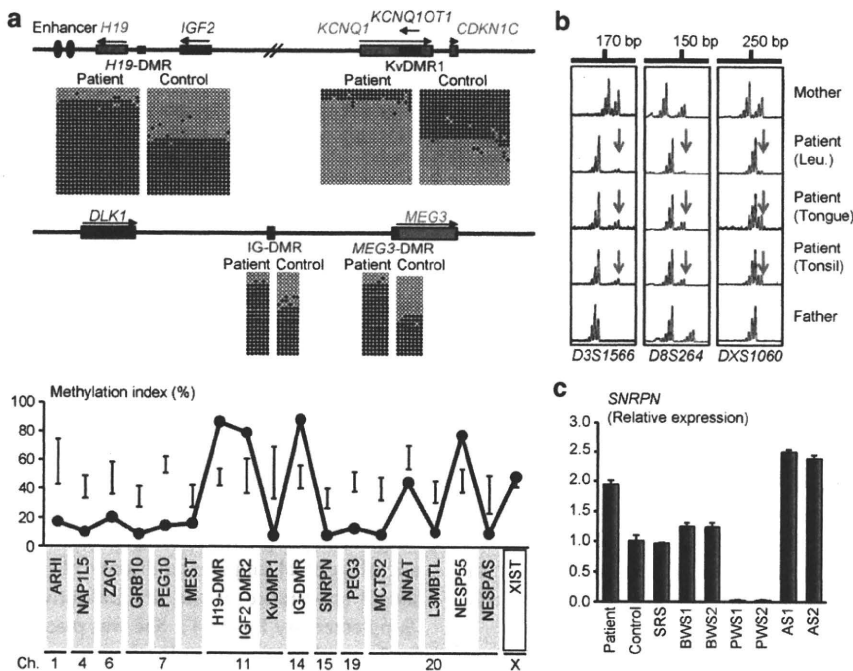


Figure 1 Representative molecular results. (a) Methylation analysis. Upper part: Bisulfite sequencing data for the *H19*-DMR and the KvDMR1 on 11p15.5, and those for the IG-DMR and the *MEG3*-DMR on 14q32.2. Each line indicates a single clone, and each circle denotes a CpG dinucleotide; filled and open circles represent methylated and unmethylated cytosines, respectively. Paternally expressed genes are shown in blue, maternally expressed gene in red, and the DMRs in green. The *H19*-DMR, the IG-DMR, and the *MEG3*-DMR are usually methylated after paternal transmission and unmethylated after maternal transmission, whereas the KvDMR1 is usually unmethylated after paternal transmission and methylated after maternal transmission.^{10,11} Lower part: Methylation indices (the ratios of methylated clones) obtained from the COBRA analyses for the 18 DMRs. The DMRs highlighted in blue and pink are methylated after paternal and maternal transmissions, respectively. The black vertical bars indicate the reference data (maximum – minimum) in leukocyte genomic DNA of 20 normal control subjects (the *XIST*-DMR data are obtained from 16 control females). (b) Representative microsatellite analysis. Major peaks of paternal origin and minor peaks of maternal origin (red arrows) have been identified in this patient. The minor peaks of maternal origin are more obvious in tongue and tonsil tissues than in leukocytes (Leu.). (c) Relative expression level (mean \pm s.d.) of *SNRPN*. The data are normalized against *TBP*. SRS: an SRS patient with an epimutation (hypomethylation) of the *H19*-DMR; BWS1: a BWS patient with an epimutation (hypermethylation) of the *H19*-DMR; BWS2: a BWS patient with upd(11)pat; PWS1: a Prader-Willi syndrome (PWS) patient with upd(15)mat; PWS2: a PWS patient with an epimutation (hypermethylation) of the *SNRPN*-DMR; AS1: an Angelman syndrome (AS) patient with upd(15)pat; and AS2: an AS patient with an epimutation (hypomethylation) of the *SNRPN*-DMR. The data were obtained using an ABI Prism 7000 Sequence Detection System (Applied Biosystems).

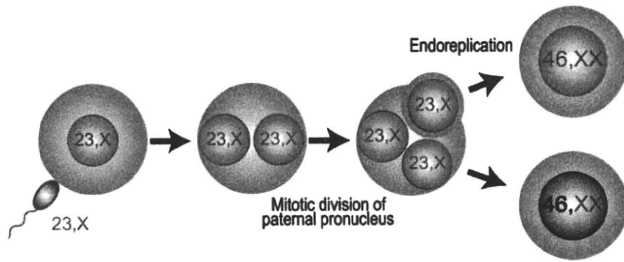


Figure 2 Schematic representation of the generation of the androgenetic/biparental mosaicism. Polar bodies are not shown.

one blastomere containing a paternally derived pronucleus and in the formation of the normal cell lineage by union of paternally and maternally derived pronuclei (Figure 2). This model has been proposed for androgenetic/biparental mosaicism generated after fertilization between a single ovum and a single sperm.^{5,15,16} The normal methylation pattern of the *XIST*-DMR is explained by assuming that the two X chromosomes in the androgenetic cell lineage undergo random X-inactivation, as in the normal cell lineage. Furthermore, the results of microsatellite analysis imply that the androgenetic cells were more prevalent in leukocytes than in tongue and tonsil tissues.

A somatic androgenetic cell lineage has been identified in seven liveborn patients including this patient (Supplementary Table 1).^{3–7} In this context, leukocytes are preferentially utilized for genetic analyses in human patients, and detailed examinations such as analyses of plural DMRs are necessary to detect an androgenetic cell lineage. Thus, the hitherto identified patients would be limited to those who had androgenetic cells as a predominant cell lineage in leukocytes probably because of a stochastic event and received detailed molecular studies. If so, an androgenetic cell lineage may not be so rare, and could be revealed by detailed analyses as well as examinations of additional tissues in patients with relatively complex phenotypes, as observed in the present patient.

Phenotypic features in androgenetic/biparental mosaicism would be determined by several factors. They include (1) the ratio of two cell lineages in various tissues/organs, (2) the number of imprinted domains relevant to specific features (for example, dysregulation of the imprinted domains on 11p15.5 and 14q32.2 is involved in placentomegaly^{9,17}), (3) the degree of clinical effects of dysregulated imprinted domains (an (epi)dominant effect has been assumed for the 11p15.5 imprinted domains¹⁸), (4) expression levels of imprinted genes in androgenetic cells (although *SNRPN* expression of this patient was consistent with androgenetic cells being predominant in leukocytes, complicated expression patterns have been identified for several imprinted genes in both androgenetic and parthenogenetic fetal mice, probably because of perturbed *cis*- and *trans*-acting regulatory mechanisms¹⁹) and (5) unmasking of possible paternally inherited recessive mutation(s) in androgenetic cells. Thus, in this patient, it appears that the extent of overall (epi)genetic aberrations exceeded the threshold level for the development of BWS-like and upd(14)pat-like body and placental phenotypes, but remained below

the threshold level for the occurrence of other imprinting disorders or recessive Mendelian disorders.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ACKNOWLEDGEMENTS

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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 mu-

tations 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; IGH, 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.

pituitary 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 *POU1F1*, *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/ese_finder.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

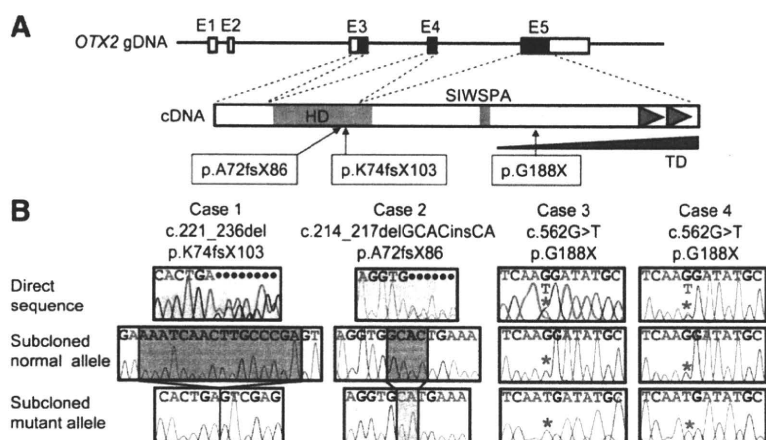


FIG. 1. Sequence analysis in cases 1–4. A, The structure of *OTX2* (the isoform-b) and the position of the mutations identified. The black and white boxes on genomic DNA (gDNA) denote the coding regions on exons 1–5 (E1–E5) and the untranslated regions, respectively. *OTX2* encodes the HD (a blue region),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

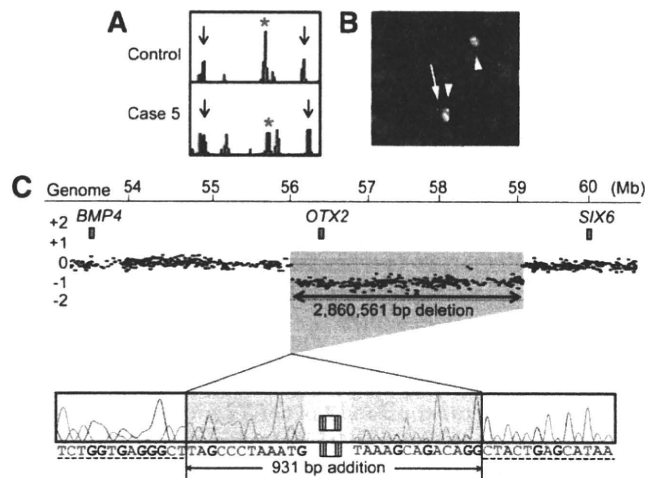


FIG. 2. Deletion analysis in case 5. **A**, MLPA analysis. The red asterisk indicates peaks for the *OTX2* exon 4, and the black arrows indicate control peaks. The red peaks indicate the internal size markers. Deletion of the MLPA probe binding site is indicated by the reduced peak height. **B**, FISH analysis. The probe for *OTX2* detects only a single red signal (an arrow), whereas the RP11-56612 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.

S, 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

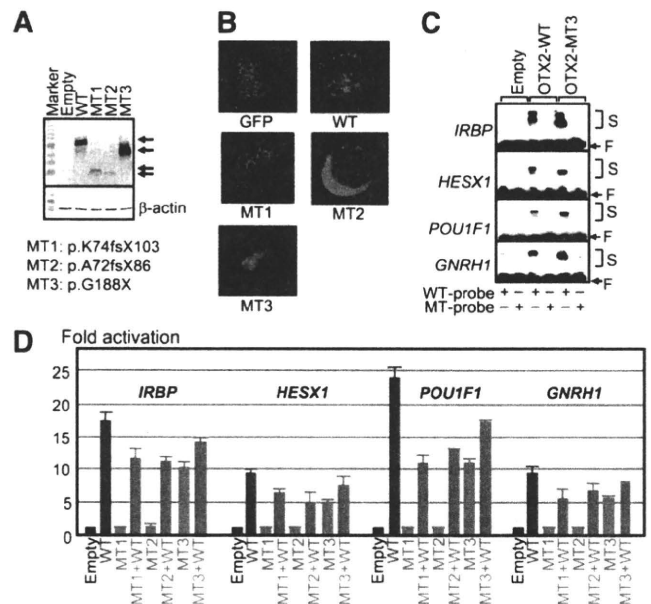


FIG. 3. Functional studies. **A**, Western blot analysis. Both WT and MT1–MT3 OTX2 proteins are detected with different molecular masses (arrows). WT, Wild type; MT1, p.K74fsX103; MT2, p.A72fsX86; and MT3, p.G188X. **B**, Subcellular localization analysis. Whereas green fluorescent protein (GFP) alone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

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

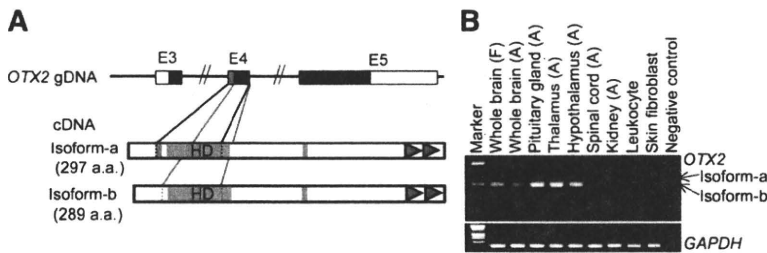


FIG. 4. PCR-based human cDNA library screening for *OTX2* (35 cycles). A, Schematic representation of the *OTX2* isoform-a (NM_21728.2) and isoform-b (NM_172337.1). Because of the two alternative splice acceptor sites at the boundary between intron 3 and exon 4, isoform-a carries eight amino acids (shown 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, hemizyosity 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	c.221_236del	c.214_217del	c.562G>T	c.562G>T	Whole gene deletion	c.402_403insC	c.674A>G	c.674A>G	c.405_406insCT
cDNA	p.K74fsX103 Severe LOF	GCACinsCA p.A72fsX86 Severe LOF	p.G188X Mild LOF	p.G188X Mild LOF	Absent	p.S135fsX136 Severe LOF	p.N225S DN	p.N225S DN	p.S136fsX178 Severe LOF
Protein Function	AO	MO	MO	MO	MO	AO	N.D.	N.D.	AO
Ocular malformation	MO	MO	MO	MO	AO	AO	N.D.	N.D.	AO
Right	+	Uncertain	+	+	+	+	N.D.	N.D.	+
Left	-	-	-	-	-	-	N.D.	N.D.	-
Developmental delay	-	-	-	-	-	-	-	-	-
Prenatal growth	-	-	-	-	-	-	-	-	-
failure ^c	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)
(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 weight (kg)	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.
Birth OFC (cm)	+	-	+	-	+	+	+	+	+
(SDS)	+	-	+	-	+	+	+	+	+
Postnatal	+	-	+	-	+	+	+	+	+
growth failure ^c	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 height (cm)	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
(SDS)	N.E.	N.E.	N.E.	N.E.	N.E.	46 (-1.9)	N.D.	N.D.	47.2 (-2.7) ^f
Present OFC (cm)	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.
(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.
Maternal height (cm)	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
Affected pituitary hormones	+	N.E.	+	N.E.	+	-	+	+	+
MRI findings	+	N.E.	+	N.E.	-	-	+	+	+
Pituitary hypoplasia	+	N.E.	+	N.E.	-	-	+	+	+
EPP	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 numbers 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.