CLINICAL REPORT

AMERICAN JOURNAL OF medical genetics PART A

Growth Hormone Secretion and its Effect on Height in Pediatric Patients With Different Genotypes of Prader–Willi Syndrome

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There have been multiple reports regarding the growth hormone (GH) secretion in patients with Prader–Willi syndrome (PWS). However, none have compared GH secretion in children with deletion group to those with maternal uniparental disomy (UPD). We evaluated the GH secretion in pediatric patients with PWS. Seventy-six patients with a deletion ($n = 55$) or UPD ($n = 21$) were studied. The secretion of GH by insulin stimulation in the patients with UPD (3.6 ± 2.2 ng/ml) was significantly lower than those with deletions (peak GH level: 11.1 ± 8.6 ng/ml; $P = 0.0015$). We also compared the response to GH replacement therapy. Yearly improvements in height standard deviation score (SDS) were similar in the two groups (first year SDS: 0.47 ± 0.47, deletion; 0.68 ± 0.26, UPD; $P = 0.14$).

Key words: Prader–Willi syndrome; growth hormone; deletion; uniparental disomy; genotype; children

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INTRODUCTION

Prader–Willi syndrome (PWS) is a pleiotropic congenital disorder [Prader et al., 1956] that includes neonatal hypotonia, hypogonadism, developmental delay, childhood-onset obesity, short stature, and behavioral abnormalities [Goldstone et al., 2008]. There have been multiple studies investigating growth hormone (GH) secretion in patients with PWS [Corrias et al., 2000; Grugni et al., 2006].

Although it was not initially clear whether GH secretion was diminished in these patients, recent studies established impaired GH secretion in patients with PWS, including low insulin like growth factor 1 (IGF-1) levels and favorable responses to GH treatment [Eiholzer et al., 2000]. Whether the degree of this GH deficiency depends on the genetic subtypes (deletion or uniparental disomy, UPD) is not known. Grugni et al. [2009] reported that GH secretion in adult patients with UPD was lower than that of those with deletions. However, they did not compare their response to GH treatment. Moreover, to our knowledge, there have been no studies to evaluate GH secretion and response to GH treatment in different genetic subtypes in children with PWS. We therefore investigated whether there were any differences in GH secretion and the response to GH treatment according to the difference of genotypes in children with PWS.

MATERIALS AND METHODS

Patients

Seventy-six Japanese patients with PWS followed at our University Hospital were included in this study. All patients had genetically confirmed PWS by methylation study, fluorescence in situ hybridization (FISH), and/or analysis of microsatellite makers and were prepubertal (Tanner breast stage 1, age <12 years for girls; testicular volume <4 ml, age <14 years for boys) at the initiation of GH. Medical records were reviewed and data including genotype, birth length, birth weight, age, height, body mass index (BMI: Weight (kg)/height (m) 2), serum IGF-1 level, and insulin-like growth factor binding protein-3 (IGFBP-3) level at the initiation of GH treatment were obtained (Table I).

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TABLE I. Baseline Characteristics of Subjects

	Deletion	UPD	
N of prepubertal PWS	55(M35, F20)	21(M12, F9)	
Birth length	47.6 ± 2.4 cm	45.3 ± 5.2 cm	$P=0.045$
Birth weight	2496 ± 379 g	2378 ± 435 g	$P=0.37$
Age at start of GH (yr)	3.5y (8m–13.5y)	3.6y (10m–11y)	
BMI at start of GH (%)	17.2 ± 4.7	17.3 ± 3.0	$P=0.97$
Height SDS_{PWS} at start	−0.52 ± 1.10	−0.72 ± 0.62	$P=0.46$
Height SDS_{NOR} at start	−2.68 ± 1.53	−2.86 ± 1.0	$P=0.64$
IGF-I at start (ng/ml)	85.1 ± 81.5	62.2 ± 63.7	$P=0.30$
IGFBP3 at start (μg/ml)	1.34 ± 0.52	1.24 ± 0.68	$P=0.56$

Height SDSPWS: Height SDS score for Japanese patients with PWS.
Height SDSNOR: Height SDS score in Japanese normal children.

Fifty-five (35 males) patients with deletions and 21 (12 males) patients with UPD were enrolled. The birth weight, age, height, BMI, serum IGF-1 level, and IGFBP-3 level at the initiation of GH therapy were similar between the two groups, whereas the birth length was lower in the UPD group than in the deletion group ($P=0.045$).

The study protocol was reviewed and approved by the Institutional Review Board of Dokkyo Medical University following the World Medical Association Declaration of Helsinki. All the participants and/or guardians provided written informed consent to participate.

Endocrine Protocol

The GH stimulation tests were performed with arginine (0.5 g/kg) intravenously or insulin (0.1 U/kg) intravenously prior to the initiation of GH treatment. Eleven patients in the deletion group and four patients in the UPD group were excluded from this study because data about GH stimulation tests were unavailable. These excluded patients had been already on GH treatment in other facilities before being referred to us. The rest of the patients underwent ariginine stimulation only (deletion, n = 3; UPD, n = 1), insulin stimulation only (deletion, n = 1; UPD, n = 0), or both arginine and insulin stimulation (deletion, n = 40; UPD, n = 16). The GH responses were evaluated by GH peak levels. To evaluate the efficacy of GH, we used the Japanese PWS height standard deviation score (SDS) and BMI [Nagai et al., 2000]. All the patients were treated with recombinant human GH (Genotropin, Pfizer, New York, NY) 0.245 mg/ kg/week subcutaneously.

Analysis

We compared GH peak levels in response to arginine and/or insulin stimulation tests in patients with deletions to that of patients with UPD. Then we compared efficacy of GH treatment between those groups by evaluating the yearly change in height SDSs and BMI. We analyzed the data for the first 3 years because previous studies showed that the efficacy of GH treatment on height is generally most prominent during early period after the initiation of growth hormone [Eiholzer and l'Allemand, 2000; Obata et al., 2003]. We used paired *t*-test, Student *t*-test, Welch *t*-test, and Mann–Whitney's U-test for statistical analyses.

RESULTS

Although responses to arginine stimulation were similar (peak GH level: 9.40 ± 6.8 ng/ml, deletion; 6.32 ± 4.60 ng/ml, UPD; $P=0.092$), responses to insulin stimulation test were significantly lower in the UPD group than in the deletion group (peak GH level: 11.1 ± 8.6 ng/ml, deletion; 3.6 ± 2.2 ng/ml, UPD; $P=0.0013$, Table II). Yearly improvements in height SDS were not significantly different between the groups (first year SDS: 0.47 ± 0.47, deletion; 0.68 ± 0.26, UPD; $P=0.14$, Table III). However, it was significantly different in the two groups when compared within the subgroups satisfying the Japanese criteria (peak GH response to at least two different provocation tests <6 ng/ml) of GH deficiency (0.42 ± 0.26, deletion; 0.70 ± 0.21, UPD; $P=0.0044$).

DISCUSSION

The aim of this study was to investigate whether there were differences in the GH secretion and in the clinical response to the GH replacement therapy in children with PWS caused by deletions compared to those with UPD.The GH response to insulin stimulation test was significantly lower in the UPD group than in the deletion group. Grugni et al. [2009] reported that GH rise by GHRH + arginine provocation was significantly higher in adults with deletions compared to adults with UPD. Our result in pediatric patients was consistent with this adult study. We demonstrated

TABLE II. Peak GH Level by Stimulation Tests

	Deletion	UPD	
Arginine stimulation (n = 60, ng/ml)	9.4 ± 6.8	6.3 ± 4.6	$P=0.092$
Insulin stimulation (n = 57, ng/ml)	11.1 ± 8.6	3.6 ± 2.2	$P=0.0013$

TABLE III. Response to GH Treatment

	Deletion	UPD	
Height $\Delta SDS_{PWS}1$ (n = 54)	0.47 ± 0.47	0.68 ± 0.26	$P = 0.14$
Height $\Delta SDS_{PWS}2$ (n = 38)	0.090 ± 0.85	0.25 ± 0.37	$P = 0.56$
Height $\Delta SDS_{PWS}3$ (n = 31)	0.091 ± 0.34	0.054 ± 0.23	$P = 0.79$

Height ΔSDSPWS1: Yearly change in Japanese PWS height standard deviation score in the first year.
Height ΔSDSPWS2: Yearly change in Japanese PWS height standard deviation score in the second year.
Height ΔSDSPWS3: Yearly change in Japanese PWS height standard deviation score in the third year.

that the GH secretion ability in the UPD group was lower than in the deletion group in pediatric patients.

We could not demonstrate a similar between-subgroup difference by arginine provocation test. The GH level in response to the insulin and arginine provocative tests are among the most frequently used parameters to assess GH secretion. However, since their effects of stimulating the secretion of GH are distinct, it is possible that the power of this study was not large enough to detect the difference in the arginine study.

The peak GH level by insulin stimulation in deletion group was at the low-normal range in our study (11.1 ± 8.6 ng/ml). A previous study also showed that GH response to insulin and arginine was low-normal to blunted in PWS and not necessarily low [Hoybye et al., 2002]. We did not use area under the curve as a parameter to assess GH response to provocation tests in this study. Koppeschaar et al., 2004 examined whether there was any advantage of using area under the curve instead of peak value of GH. They showed that they were both similarly effective for diagnosis of adult GH deficiency.

If there is a genotype-dependent difference in the degree of GH deficiency, it is assumed that there might also be a genotype-dependent difference in the response to the GH treatment.

Therefore, we also evaluated the effect of GH on height gain in children with PWS. We hypothesized that GH efficacy was more prominent in the UPD group. However, our study showed no significant difference in yearly improvements in height SDS between the deletion and UPD group at least for the first 3 years.

It is not clear why patients with PWS due to a deletion showed similar yearly growth rate compared with those with UPD even though the GH provocative response was different. Carrascosa et al. [2011] examined the relationship of the GH provocative test to growth on GH therapy and found no significant relationship in prepubertal short children. Previous studies suggested that there may be considerable heterogeneity in GH secretion in patients with PWS [Burman et al., 2001; Grugni et al., 2009]. Indeed, the overall comparison of the deletion and UPD groups in our study did not show a significant difference in the response to GH therapy. However, when compared within subpopulation of patients showing overt GH deficiency alone, the UPD group showed significantly greater response to GH therapy than deletion group. Moreover, polymorphism in the GH receptors might play a role in the heterogeneous responses to the GH treatment. Park et al. [2011] reported that patients with PWS and an exon-3 deletion polymorphism (d3, Database of Genomic Variants ID: Variation_64191) in the GH receptor gene had significantly greater height SDSs and higher IGF-I level before GH treatment. Further study with a larger number of patients is needed to compare the response to GH treatment among genotypes in the subgroups stratified by factors including severity of GH deficiency and receptor polymorphism.

In conclusion, the secretion of GH in the pediatric patients with UPD group was significantly lower than that in those with deletion group. However, yearly improvements in height SDS by GH treatment were not significantly different between the deletion and UPD group.

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RESEARCH ARTICLE

AMERICAN JOURNAL OF medical genetics PART A

Complex Genomic Rearrangement in the *SOX9* 5′ Region in a Patient With Pierre Robin Sequence and Hypoplastic Left Scapula

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Pierre Robin sequence (PRS) can occur as a component of campomelic dysplasia (CD) and acampomelic CD (ACD) caused by dysfunction or dysregulation of SOX9, although it can also take place as an isolated form. Recently, genomic alterations in the far upstream and the far downstream region of *SOX9* have been identified in patients with isolated PRS. Here, we report on a male patient with PRS and a heterozygous genomic rearrangement in the 5′ region of *SOX9*. Clinical analysis revealed PRS-compatible craniofacial anomalies, mild hypoplasia of the left scapula, and normal male external genitalia. Molecular analysis identified a paracentric inversion on the long arm of chromosome 17 with breakpoints at 17q21.31 and 17q24.3, and a microdeletion spanning from −4.15 to −1.16 Mb relative to *SOX9*. These findings indicate that the chromosomal region more than 1.16 Mb apart from *SOX9* contains at least one developmental enhancer(s) for *SOX9* that plays a critical role in the development of the mandible and a relatively small role in the development of the scapula. Moreover, the concept of exclusion mapping argues that putative CD/ACD loci are located within the 1.16 Mb region closest to *SOX9* coding exons, which remain intact in this Non-CD/ACD patient. This study provides a novel example for long-range *cis*-regulatory mutations of *SOX9*. © 2012 Wiley Periodicals, Inc.

Key words: campomelic dysplasia; deletion; inversion; enhancer; noncoding element

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INTRODUCTION

Pierre Robin sequence (PRS) (OMIM 261800) is a congenital malformation sequence characterized by micrognathia, glossoptosis, and posterior U-shaped cleft palate [Robin, 1934]. The primary defect of PRS is assumed to be mandibular hypoplasia caused by impaired chondrogenesis or aberrant proliferation of neural crest cells [Gordon et al., 2009]. PRS frequently occurs as a component of known syndromes such as campomelic dysplasia (CD) (OMIM 114290), acampomelic CD (ACD), and Stickler syndrome (OMIM 108300), although PRS can also take place as an isolated (nonsyndromic) form [Holder-Espinasse et al., 2001].

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CD and ACD are caused by dysfunction or dysregulation of SOX9; multiple intragenic mutations of *SOX9* as well as various types of chromosomal rearrangements around the coding exons have been identified in patients with CD and ACD [Meyer et al., 1997; Gordon et al., 2009]. In addition to PRS, patients with CD manifest bowing of the long bones (campomelia), hypoplastic scapulae, pelvic malformations, a missing pair of ribs, clubfeet, and 46,XY gonadal dysgenesis. ACD represents a mild variant of CD lacking campomelia. Since PRS is present in most patients with CD and ACD [Gordon et al., 2009], SOX9 likely plays a particularly important role in the development of the mandible.

Recently, molecular defects in the far upstream and the far downstream region of *SOX9* have been identified in patients with isolated PRS. Jamshidi et al. [2004] and Jakobsen et al. [2007] identified balanced translocations of t(2;17) in familial and sporadic PRS cases, respectively, and found that the 17q breakpoints are located more than 1.0 Mb upstream of *SOX9*. Subsequently, Benko et al. [2009] identified variable genomic abnormalities (translocations, deletions, and a nucleotide substitution) at a position more than 1.0 Mb apart from *SOX9* in two sporadic and five familial cases with PRS. Furthermore, Benko et al. [2009] showed that the deletions and translocations included several highly conserved noncoding elements (HCNE) and the nucleotide substitution abolished the tissue-specific enhancer activity of one of these HCNEs (HCNE-F2). These data provide the first evidence that dysfunction of the very-long-range enhancer(s) of *SOX9* causes isolated PRS. However, there is no other report of patients with a molecular defect in the far upstream or the far downstream region of *SOX9*. Here, we report on a male patient with a complex genomic rearrangement in the 5′ region of *SOX9*. Clinical and molecular analyses of this patient provide further information on tissue-specific regulation of *SOX9*.

CLINICAL REPORT

This Japanese male was born at 38 weeks of gestation after an uncomplicated pregnancy and delivery. At birth, his length was 48.0 cm (−0.48 SD), weight 2.83 kg (−0.55 SD), and head circumference 32.0 cm (±0 SD). Immediately after birth, he was referred to our clinic because of respiratory distress and facial anomalies. He had hypoplastic mandible, cleft palate, and glossoptosis and was therefore diagnosed as having PRS. In addition, he showed bilateral clubfeet. Campomelia and tibial skin dimples were not observed.

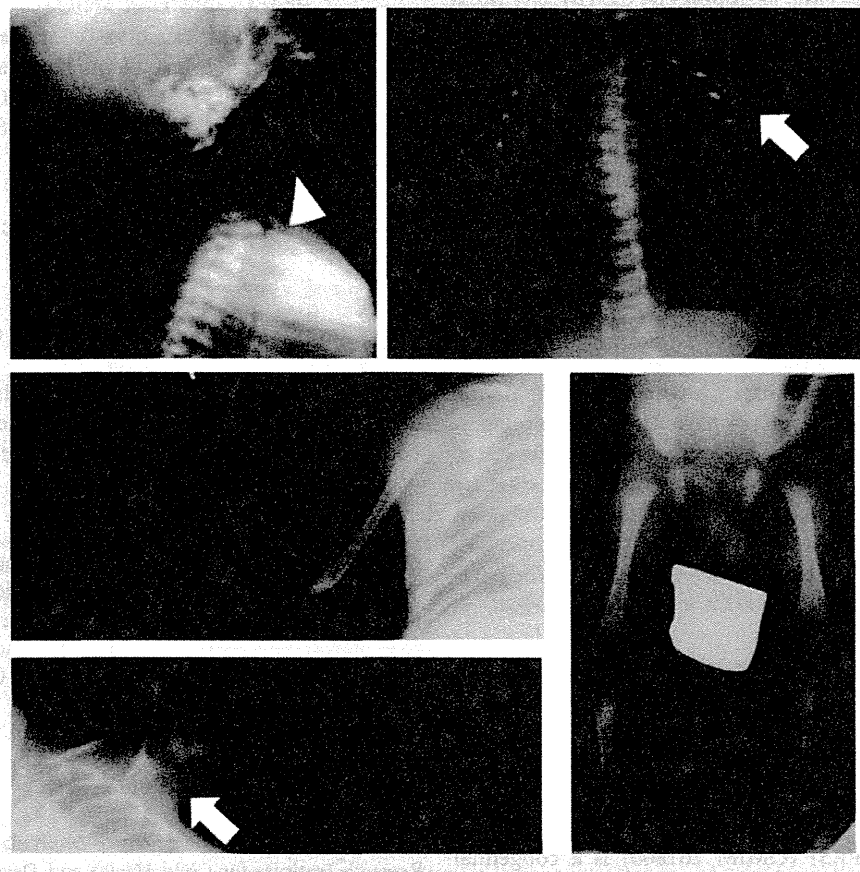

FIG. 1. Roentgenograms of the patient at 2 weeks of age. Mild hypoplasia of left scapula (white arrows) and micrognathia (a white arrowhead) are indicated.

He manifested normal male external genitalia with bilateral descended testes. On skeletal survey, dolichocephaly with hypoplasia of the facial bone, micrognathia, and hypoplasia of the left scapula were evident (Fig. 1). The right scapula was unremarkable. The ischia appeared somewhat broad, and the ischiopubic synchondroses wide; yet, these findings were too mild to be distinguishable from the normal range. Other radiological hallmarks in CD, such as cervical kyphosis, hypoplastic pedicles of the thoracic spine, and narrow ilia, were not discernible. G-banding chromosome analysis showed a normal 46,XY karyotype. Direct sequence analysis for *SOX9* detected no mutation in the coding region [Wada et al., 2009].

During several months after birth, he continually required medical intervention for respiratory and feeding difficulties. He underwent a tracheotomy at 8 months of age. He showed no obvious developmental delay; he was able to stand and walk along the wall at 1 year of age and was able to indicate his desires and needs by pointing at 1 year and 7 months of age. On his last examination at 1 year and 7 months of age, he measured 76.3 cm (−1.77 SD) and weighed 9.2 kg (−1.31 SD). His parents and sister were clinically normal.

MOLECULAR ANALYSES

This study was approved by the Institutional Review Board Committee at the National Center for Child Health and Development. After obtaining written informed consent from the parents, a peripheral blood sample was taken from the patient. Parental samples were not available for molecular analysis.

High-resolution chromosomal banding revealed a karyotype of 46,XY,der(17)inv(17)(q21.31q24.3)del(17)(q24.3q23?) (Fig. 2A). Fluorescence in situ hybridization (FISH) analysis using RP11-84E24-BAC containing *SOX9* and RP11-20N01-BAC on 17q21.31 indicated a paracentric inversion on one of the two chromosome 17 (Fig. 2B and C). Signals for *SOX9* were detected on two chromosome 17. Comparative genomic hybridization (CGH) analysis using a human genome oligoarray (1 × 1 M format, G4447A, Agilent Technologies, Palo Alto, CA) indicated a heterozygous deletion in the *SOX9* upstream region (Fig. 3A). In silico analysis using UCSC genome browser (http://genome.ucsc.edu/; hg 19; NCBI Build 37) showed that the deletion was 2.99 Mb in physical length and flanked by the proximal and the distal breakpoints residing at −4.15 and −1.16 Mb to *SOX9*, respectively. A total of 18 known genes were located within the deleted region, as assessed using the Refseq database (Fig. 3A).

DISCUSSION

A complex genomic rearrangement in the 5′ region of *SOX9* was identified in a boy with PRS. The genomic lesion started at a point 1.16 Mb upstream of *SOX9* and affected several HCNEs. In particular, HCNE-F2, previously shown to act as a developmental enhancer for the craniofacial region [Benko et al., 2009], was deleted in this patient (Fig. 3B). Thus, the PRS phenotype of this patient

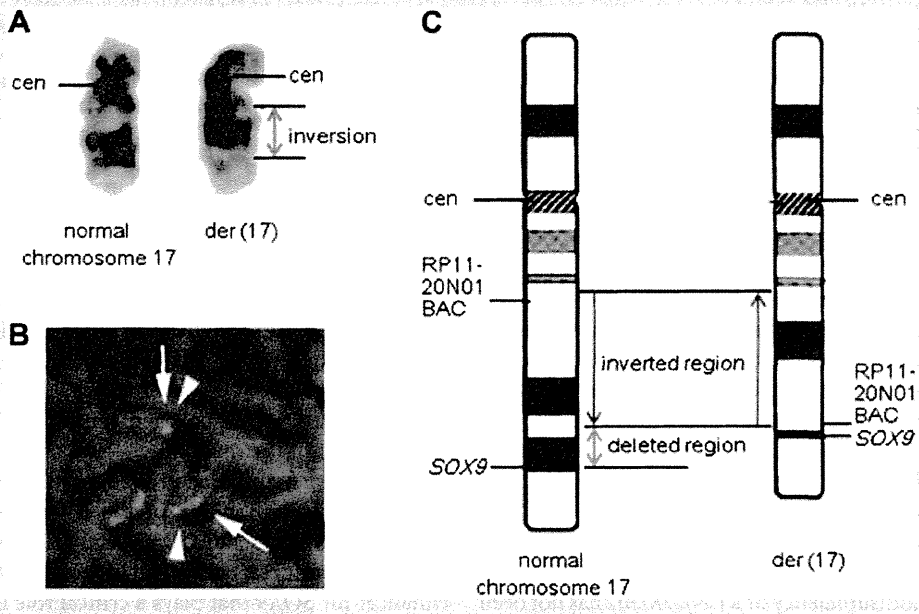

FIG. 2. Chromosomal banding and FISH analysis. A: High-resolution chromosomal banding indicating the presence of a deletion and an inversion on the long arm of chromosome 17. cen, centromere. B: Representative results of FISH analysis. The arrowheads denote RP11-84E24-BAC containing *SOX9* (green signals); the arrows indicate RP11-20N01-BAC on 17q21.31 (red signals). Two signals of an apparently different distance are present on two chromosome 17, indicating an inversion on one of two chromosome 17. Signals for *SOX9* are normally present on both chromosome 17. C: Schematic representation of the genomic rearrangement of the patient.

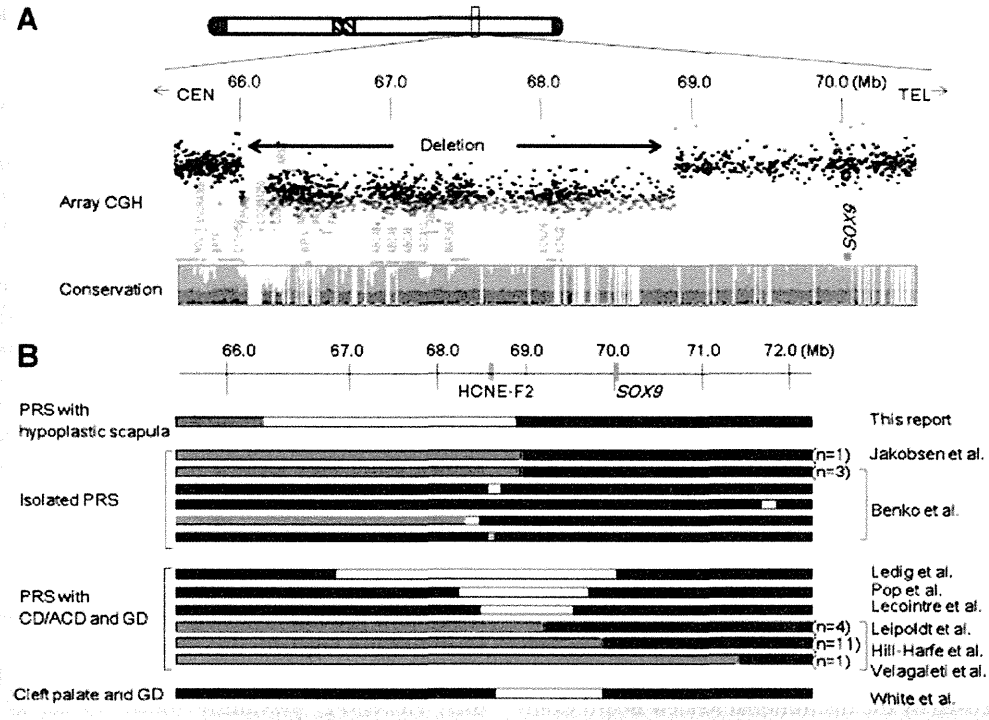

FIG. 3. Genomic abnormalities around *SOX9*. A: Oligoarray CGH analysis in the patient. The black, the red, and the green dots denote signals indicative of the normal, the increased (>+0.5), and the decreased (<−1.0) copy numbers, respectively. The deletion is 2.99 Mb in length and encompasses 18 Refseq genes and several highly conserved noncoding elements. The proximal border of the deletion is located at a point 1.16 Mb upstream of *SOX9*. Genomic positions are referred to the Human Genome (February 2009, hg 19; NCBI Build 37). B: Schematic representation of genomic lesions and clinical features of present case and previously reported patients [Pop et al., 2004; Hill-Harfe et al., 2005; Velagaleti et al., 2005; Jakobsen et al., 2007; Leipoldt et al., 2007; Benko et al., 2009; Lecointre et al., 2009; Ledig et al., 2010; White et al., 2011]. The white areas denote monosomic regions and the black areas, the disomic regions. The purple area indicates the inverted region. The blue regions in translocation-positive patients indicate DNA sequences derived from other chromosomes; the approximate location of translocation breakpoint clusters are shown in green, and the number of breakpoints within each cluster is shown in parenthesis. The gray region depicts a dosage-unknown region. The orange dot denotes a nucleotide substitution. HCNE-F2, the highly conserved noncoding element with enhancer activity reported by Benko et al. [2009]; PRS, Pierre Robin sequence; ACD, acampomelic campomelic dysplasia; GD, gonadal dysgenesis; CD, campomelic dysplasia.

would be ascribed to *SOX9* misexpression due to loss of HCNE-F2, although we cannot exclude the possibility of another hitherto unidentified *cis*-regulatory element(s) of *SOX9* being affected by the deletion/inversion. In this regard, while the deletion removed 18 genes, clinical features of the patient can be explained by *SOX9* dysfunction alone. Moreover, none of the 18 genes, except for *KCNJ2*, are known to be involved in mandibular growth. Furthermore, whereas dominant negative mutations of human *KCNJ2* as well as homozygous deletion of mouse *KcnJ2* have been shown to result in cleft palate and micrognathia [Zaritsky et al., 2000; Andelfinger et al., 2002], haploinsufficiency of *KCNJ2/KcnJ2* has not been shown to cause such abnormalities. Hence, the patient represents a novel case with PRS caused by a *SOX9 cis*-regulatory mutation. Such submicroscopic genomic rearrangements may also be present in other patients with isolated PRS. Indeed, only a few genes have been identified as causative genes for isolated PRS. In this regard, it is noteworthy that mutations of collagen genes including *COL11A2* and *COL11A1* have been shown to cause a PRS as a component of Stickler syndrome without apparent ocular involvement [Vikkula et al., 1995; Annunen et al., 1999]. Since collagen genes are known to be direct targets of SOX9 [Gordon et al., 2009], these data suggest that transactivation of collagen genes by SOX9 is critical for the development of the mandible.

This patient manifested PRS-compatible craniofacial abnormalities and mild hypoplasia of the left scapula. Nevertheless, he showed no typical CD/ACD skeletal features. These data indicate that the genomic rearrangement of the patient disrupted at least one enhancer for *SOX9* that plays a critical role in the development of the mandible and a small role in the development of the scapula. In addition, the concept of exclusion mapping implies that tissue-specific enhancers for long bones, pelvic bones, and ribs are located within the 1.16 Mb region closest to *SOX9*, because CD/ACD is known to be a fully penetrant phenotype in patients with intragenic mutations of *SOX9* [Meyer et al., 1997]. Consistent with this,

previous studies have suggested that putative loci for CD/ACD are located within the 1.0 Mb region from *SOX9* [Gordon et al., 2009]. Nevertheless, the phenotype of this patient could also be explained by assuming that there is a global developmental enhancer(s) of *SOX9* in the region more than 1.16 Mb apart from *SOX9* and that the mandible and the scapula are more sensitive to reduced transcriptional levels of *SOX9* than other skeletal tissues [Gordon et al., 2009]. Indeed, various skeletal changes of the patient such as clubfeet, borderline broad ischia, and relatively wide ischiopubic synchondroses, may be related to mildly impaired *SOX9* expression. In this context, it is noteworthy that CD, ACD, and isolated PRS are currently regarded as a continuum of a disorder caused by *SOX9* abnormalities [Gordon et al., 2009]. Thus, this patient may represent an intermediate phenotype between ACD and isolated PRS.

This patient had normal male external genitalia, indicating that the testis-specific enhancer(s) of *SOX9* is preserved in this patient. Consistent with this, previous studies on translocation-positive patients suggested that a testis-specific enhancer(s) is located within the 789 kb region closest to *SOX9* [Gordon et al., 2009]. Moreover, animal studies have identified a testis-specific enhancer immediately upstream of *Sox9* [Sekido and Lovell-Badge, 2008]. However, fairly well preserved masculinization of this patient may be ascribed to incomplete penetrance of gonadal dysgenesis in *SOX9* abnormalities, because normal testicular development has been observed in about 25% of 46,XY individuals with a *SOX9* intragenic mutation [Mansour et al., 1995].

To date, various types of cryptic deletions have been identified in patients with PRS (Fig. 3B). Notably, there is no overlapping region of deletion that is shared by all PRS cases, although the deletions of sporadic case 4 and familial case 1 reported by Benko et al. [2009] are located within the deleted region of the patient described herein. These results imply that multiple *cis*-acting elements around *SOX9* are required for the appropriate development of the mandible. Further analysis in a large cohort of PRS patients would enable us to clarify the precise locations of *SOX9* tissue-specific enhancers. In this regard, array CGH would serve as a powerful tool for screening of such patients, because it can detect various copy number alterations in a chromosomal region of several megabases.

In summary, the present study provides a novel example for long-range *cis*-regulatory mutations of *SOX9*. Our findings suggest that the genomic region more than 1.16 Mb upstream of *SOX9* includes at least one *cis*-acting element that regulates *SOX9* expression in the developing mandible, and, to a lesser extent, in the developing scapula. Further studies will permit the full characterization of the genomic environment involved in tissue-specific regulation of *SOX9*.

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ORIGINAL ARTICLE

Association of variants in genes involved in environmental chemical metabolism and risk of cryptorchidism and hypospadias

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We hypothesized that single-nucleotide polymorphisms (SNPs) of genes involved in environmental endocrine disruptors (EEDs) metabolism might influence the risk of male genital malformations. In this study, we explored for association between 384 SNPs in 15 genes (*AHR*, *AHRR*, *ARNT*, *ARNT2*, *NR1I2*, *RXRA*, *RXRB*, *RXRG*, *CYP1A1*, *CYP1A2*, *CYP1B1*, *CYP2B6*, *CYP3A4*, *CYP17A1* and *CYP19A1*) and risk of cryptorchidism (CO) and hypospadias (HS) in 334 Japanese (JPN) males (141 controls, 95 CO and 98 HS) and 187 Italian (ITA) males (129 controls and 58 CO). In the JPN study group, five SNPs from *ARNT2* (*rs2278705* and *rs5000770*), *CYP1A2* (*rs2069521*), *CYP17A1* (*rs4919686*) and *NR1I2* (*rs2472680*) were significantly associated at both allelic and genotypic levels with risk of at least one genital malformation phenotype. In the ITA study group, two SNPs in *AHR* (*rs3757824*) and *ARNT2* (*rs1020397*) were significantly associated with risk of CO. Interaction analysis of the positive SNPs using multifactor dimensionality reduction demonstrated that synergistic interaction between *rs2472680*, *rs4919686* and *rs5000770* had 62.81% prediction accuracy for CO ($P=0.011$) and that between *rs2069521* and *rs2278705* had 69.98% prediction accuracy for HS ($P=0.001$) in JPN population. In a combined analysis of JPN and ITA population, the most significant multi-locus association was observed between *rs5000770* and *rs3757824*, which had 65.70% prediction accuracy for CO ($P=0.055$). Our findings indicate that genetic polymorphisms in genes involved in EED metabolism are associated with risk of CO and HS.
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Keywords: cryptorchidism; cytochrome P450; endocrine disruptor; hypospadias; multifactor dimensionality reduction; nuclear receptor; SNP

INTRODUCTION

During the early stages of development (embryonic, fetal and infant), humans are highly vulnerable to environmental hazards. It has been proposed that *in utero* exposure to environmental endocrine disruptors (EEDs) could adversely affect fetal growth and induce several types of male genital malformation (MGM), such as cryptorchidism (CO) and hypospadias (HS).[1,2] However, epidemiological studies on this issue have produced conflicting results.[3–5] The effect of EEDs would depend on several factors, including the dosage of EED exposure, the developmental stage in which EED exposure occurred and inter-individual variability in genetic susceptibility to the effects of EED exposure.

The etiology of MGM seems to be multifactorial, involving genetic, hormonal and environmental factors. Single-nucleotide polymorphism (SNP) analyses have been undertaken in human populations and have identified multiple genetic variants that are linked with the prevalence of MGMs. The majority of the previous studies have been performed to exploit polymorphisms in sex hormone and endocrine-related genes, such as insulin-like factor 3 (*INSL3*), INSL3 receptor (*LGR8* or *GREAT*), androgen receptor, estrogen receptors 1 and 2 (*ESR1* and *ESR2*), steroid-5α-reductase, mastermind-like domain containing 1 (*Cxorf6*), activating transcription factor 3, fibroblast growth factor 8 and FGF receptor 2.[6–12]

However, few of these studies have focused on polymorphisms in genes involved in drug metabolism that might influence individual susceptibility to exogenous agents such as EEDs. It is well known that both the metabolism of EEDs and male sexual differentiation are mediated by a series of transcription factors and cytochrome P450

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(CYP) enzymes. Genetic polymorphisms in these transcription factors and enzymes may be important in determining individual susceptibility to EED exposure and also in the development of MGMs.[13–14] Both our study, and other previous studies have identified that genetic variants for *ESR1* and *ESR2* could raise the susceptibility of CO and HS by enhancing the effects of estrogenic EEDs, which are known as xenoestrogens and currently the largest group of known EEDs.[7,15] In addition, several nuclear receptors, such as aryl-hydrocarbon receptor (AHR) and pregnane X receptor (PXR or NR1I2), are known to be crucial for EED-mediated CYP transcription.[16] Previous studies have reported that polymorphisms in *AHR* may affect AHR functions, notably the induction of *CYP1* genes, suggesting a potential role for nuclear receptor polymorphisms in the variable responses to xenobiotic chemicals.[17] It is likely that further investigations of genetic polymorphisms involved in drug metabolism will shed increased light on the link between EED exposure and the development of MGMs.

Therefore, the aim of this study was to determine whether SNPs in genes involved in the metabolism of EEDs are associated with risk of CO and HS.

MATERIALS AND METHODS

Study populations

We conducted a case–control study in Japanese (JPN) and Italian (ITA) populations. The JPN study was based on a total of 334 genomic DNA samples collected at the Department of Molecular Endocrinology, National Research Institute for Child Health and Development, Tokyo, Japan, during the period 2002–2009. Samples were obtained from 193 male patients, aged 1–13 years; this group included 95 CO patients and 98 HS patients; samples were also obtained from 141 control males, consisting of 75 boys, aged 4–16 years, with normal external genitalia and 66 adults, aged 24–50 years, with proven fertility. The ITA study was based on a total of 187 genomic DNA samples collected at the Department of Pediatrics, University Hospital of Santa Chiara, Pisa, Italy, during the period 2006–2007. These samples were obtained from 58 CO patients, aged 1.0–2.2 years (median age 1.3 years), and 129 control males (median age 7.3 years).

All samples were obtained after written informed consent to participation in the study had been given.This study was approved by the Institutional Ethics Committees at the National Research Institute for Child Health, Japan and Development and National Institute for Environmental Studies, Japan.

Gene selection

KeyMolnet, a knowledge-based information system developed by the Institute of Medicinal Molecular Design Inc., Tokyo, Japan, was used to identify the molecular interactions of four nuclear receptors (AHR, PXR or NR1I2, ESR1 and ESR2). KeyMolnet is a bioinformatics database composed of manually curated information on relationships among human genes, molecules, diseases, pathways and drugs from selected review articles, literature and public databases. It can generate networks from any molecule and can connect the networks to biological phenomena, and to drug and disease information.[18] From the generated network, CYP enzymes that are involved in the steroid hormone biosynthesis pathway were extracted for further analysis.

SNP selection

Selection of SNPs for use in this study was based on minor allele frequencies in the JPN populations with a location more than 60 kb distance from a range lying from 20 kb upstream of transcription to 10 kb downstream of each gene. They included known tagging SNPs, which are composed of a haplotype block.

Genotyping

The concentrations of the genomic DNA samples were determined with the PicoGreen dsDNA Quantitation kit according to manufacturer's protocol (Invitrogen, Carlsbad, CA, USA). SNPs were determined using the GoldenGate assay, which uses a human BeadArray technique (Illumina, San Diego, CA, USA), and allele-specific fluorescence signals were scanned using a BeadScan500 (Illumina).

Statistical analysis

Genotype frequencies in controls were tested for concordance with the Hardy–Weinberg equilibrium using GeneSpring software, version 11.5 (Silicon Genetics, Redwood City, CA, USA). Differences in all genotype frequencies between cases and controls were tested for each SNP. Odds ratios (ORs) for disease risk and corresponding 95% confidence intervals (CIs) were calculated at the genotypic level. The Cochran–Armitage trend test corrected with Benjamini–Hochberg false discovery rate and Fisher's exact test at the genotypic level were performed using GeneSpring software, version 11.5 (Silicon Genetics). In addition, multifactor dimensionality reduction (V2.0 Beta 8.4) analysis was performed to evaluate and validate main effects associated with the risk of CO and HS using a software package freely available online (www.epistasis.org). This algorithmic tool is a nonparametric (does not assume any statistical model) and model-free (no assumption mode of genetic inheritance) exploratory method, which has been developed to detect and characterize high-order gene–gene and gene–environment interactions in studies with relatively small sample size.[19,20] Models are evaluated on the testing balanced accuracy, the cross-validation consistency and the statistical significance of the model. The testing balanced accuracy measures how often individuals are correctly classified with respect to their case/control status, and the cross-validation consistency evaluate the consistency with which individuals are classified.[21] $P<0.05$ was considered statistically significant in this study.

RESULTS

Gene and SNP selection

The molecular network was generated around four starting molecules (AHR, PXR or NR1I2, ESR1 and ESR2) within one path of both upstream and downstream from the starting point molecules by bioinformatics database tool (Figure 1). In addition to the four starting molecules, the generated network includes aryl-hydrocarbon receptor repressor (AHRR), aryl-hydrocarbon receptor nuclear translocator (ARNT), ARNT2, retinoid X receptor (RXR), and its three subtypes, RXRA, RXRB and RXRG, and 18 CYP enzymes. CYP enzymes involved in the steroid hormone biosynthesis pathway, which have been recognized as important targets for the actions of EEDs,[22] namely CYP1A1, CYP1A2, CYP1B1, CYP2B6, CYP3A4, CYP17A1 and CYP19A1, were selected for further analysis. Therefore, a total of 15 genes were selected as target genes for analyzing SNPs in this study (Table 1a and b). With the exception of RXRB, which had no tagging SNP, a total of 384 SNPs were detected in the remaining 14 genes.

Polymorphisms and CO risk in the JPN study

SNPs found to be associated with risk of CO in the JPN population are shown in Table 2. The minor homozygous *rs5000770* (AA) of *ARNT2*, heterozygous *rs4919686* (AC) of *CYP17A1* and heterozygous *rs247280* (AG) of *NR1I2* were more frequently found in the 95 CO patients than in the 141 controls (OR = 3.5, 95% CI = 1.7–7.3; OR = 3.3, 95% CI = 1.4–7.8; and OR = 2.2, 95% CI = 1.0–5.0, respectively). Furthermore, the allele frequencies of these SNPs differed significantly between the CO patients and the controls ($P_{trend}<0.05$).

Polymorphisms and HS risk in the JPN study

The SNPs found to be associated with risk of HS in the JPN study group are shown in Table 3. Minor homozygous and heterozygous *rs2069521* (AA and AG, respectively) of *CYP1A2* and minor homozygous *rs2278705* (AA) and minor homozygous *rs5000770* (AA) of *ARNT2* were more frequently found in the 98 HS patients than in the 141 controls (OR = 4.5, 95% CI = 9.3–194.6; OR = 3.7, 95%

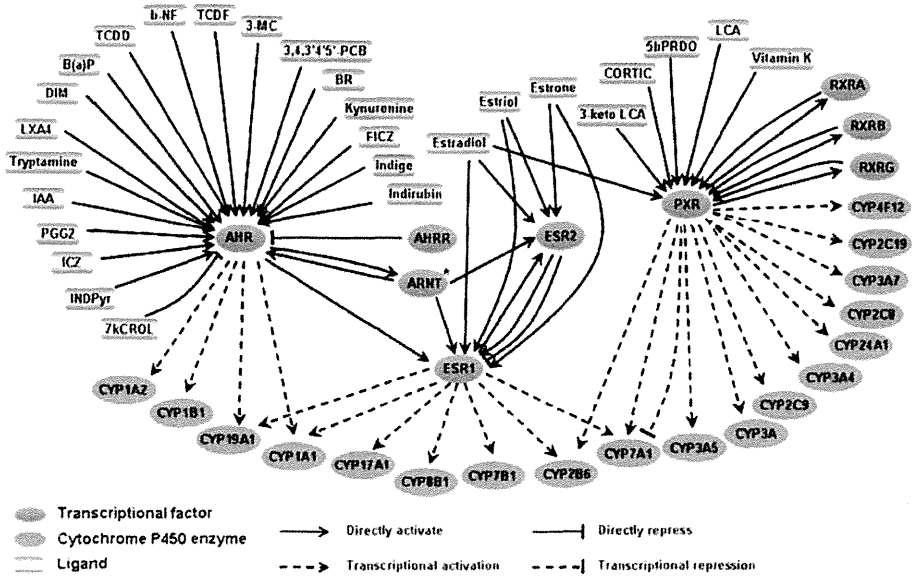

Figure 1 Network-based analysis for molecular interactions of AHR, PXR, ESR1 and ESR2 using KeyMolnet. A gene list of AHR, PXR, ESR1 and ESR2 was imported into KeyMolnet that generated a molecular network composed of 27 ligands (red), 9 transcription factors (green) and 18 cytochrome P450 enzymes (blue). Solid lines with an arrowhead and stop indicate direct activation and repression, respectively, including binding or phosphorylation. Dashed line with arrow and stop indicates stimulation and inhibition of gene expression, respectively. Asterisk (*) indicates ARNT2 in some cases according to the tissue-specific expression of ARNT and ARNT2.

Table 1

Gene symbol	*Aliases*	*Gene name*	*Sequence accession no.*	*Molecular function*	*SNP*[a]	*tgSNP*[b]
(a) List of transcription factor genes and numbers of SNPs determined in this study						
AHR		Aryl-hydrocarbon receptor	L19872, NM_001621	Nuclear receptor	17	10
AHRR	KIAA1234	Aryl-hydrocarbon receptor repressor	AB033060, NM_020731	Nuclear receptor coactivator	29	14
ARNT	HIF-1beta	Aryl-hydrocarbon receptor nuclear translocator	AF001307	Nuclear receptor coactivator	31	6
ARNT2	KIAA0307, bHLHe1	Aryl-hydrocarbon receptor nuclear translocator 2	AB002305	Nuclear receptor coactivator	69	32
NR1I2	ONR1, PXR, BXR, SXR, PAR2	Nuclear receptor subfamily 1, group I, member 2	AF061056	Nuclear receptor	21	14
RXRA	NR2B1	Retinoid X receptor, alpha	X52773	Nuclear receptor	46	19
RXRB	NR2B2, H-2RIIBP, RCoR-1	Retinoid X receptor, beta	M84820	Nuclear receptor	0	0
RXRG	NR2B3	Retinoid X receptor, gamma	U38480, NM_006917	Nuclear receptor	34	19
(b) List of CYP enzyme genes and numbers of SNPs determined in this study						
CYP1A2	P3-450, CP12	Cytochrome P450, family 1, subfamily A, polypeptide 2	AF182274, NM_000761	Drug metabolism enzyme; steroid hormone biosynthesis enzyme	10	2
CYP1B1	CP1B	Cytochrome P450, family 1, subfamily B, polypeptide 1	U56438, NM_000104	Drug metabolism enzyme; steroid hormone biosynthesis enzyme	20	6
CYP2B6	CPB6, CYPIIB6, CYP2B	Cytochrome P450, family 2, subfamily B, polypeptide 6	AF182277, NM_000767	Drug metabolism enzyme; steroid hormone biosynthesis enzyme	13	9
CYP3A4		Cytochrome P450, family 3, subfamily A, polypeptide 4	AF280107	Drug metabolism enzyme; steroid hormone biosynthesis enzyme	27	1
CYP17A1	P450C17, CPT7, S17AH	Cytochrome P450, family 17, subfamily A, polypeptide 1	M19489, NM_000102	Drug metabolism enzyme; steroid hormone biosynthesis enzyme	18	4
CYP19A1	ARO, P-450AROM, CPV1, ARO1, CYAR	Cytochrome P450, family 19, subfamily A, polypeptide 1	D14473	Drug metabolism enzyme; steroid hormone biosynthesis enzyme	40	21

[a]Number of single-nucleotide polymorphisms (SNPs).
[b]Number of tagging SNPs (tgSNPs).

Table 2 Effect of *ARNT2*, *CYP17A1* and *NR1I2* polymorphisms on the risk of CO in the JPN study group

			Case (n = 95)		*Control (n = 141)*			
Gene	*SNP*	*Genotype*	*No.*[a]	*%*	*No.*	*%*	*OR (95% CI)*	P_{trend}[b]
ARNT2	rs2278705	GG	66	70.21	104	74.28	Reference	0.141421
		AA	9	9.58	4	2.86	3.5(1.0–12.0)*	
		AG	19	20.21	32	22.86	0.9(0.5–1.8)	
	rs5000770	GG	40	42.11	78	55.32	Reference	0.002392#
		AA	27	28.42	15	10.64	3.5(1.7–7.3)*	
		AG	28	29.47	48	34.04	1.1(0.6–2.1)	
	rs7183507	GG	60	63.83	97	68.79	Reference	0.121297
		AA	6	6.38	1	0.71	9.7(1.1–82.6)*	
		AG	28	29.79	43	30.50	1.1(0.6–1.9)	
	rs7178949	AA	58	63.04	97	68.79	Reference	0.0744634
		GG	7	7.61	1	0.71	11.7(1.4–97.6)*	
		AG	27	29.35	43	30.50	1.1(0.6–1.9)	
	rs11072922	GG	55	57.89	82	58.16	Reference	0.160607
		AA	14	14.74	4	2.84	5.2(1.6–16.7)*	
		AG	26	27.37	55	39.00	0.7(0.4–1.3)	
CYP17A1	rs4919686	AA	75	81.52	131	93.57	Reference	0.0114102#
		CC	0	0	0	0		
		AC	17	18.48	9	6.43	3.3(1.4–7.8)*	
	rs6163	AC	31	39.74	72	51.43	Reference	0.0674834
		AA	13	16.67	28	20.00	1.1(0.5–2.4)	
		CC	34	43.59	40	28.57	2.0(1.1–3.7)*	
NR1I2	rs1403526	AG	26	30.23	66	46.81	Reference	0.697065
		AA	40	46.51	51	36.17	2.0(1.1–3.7)*	
		GG	20	23.26	24	17.02	2.1(1.0–4.5)*	
	rs2472680	GG	76	82.61	128	91.43	Reference	0.0436642#
		AA	0	0	0	0		
		AG	16	17.39	12	8.57	2.2(1.0–5.0)*	

Abbreviations: CI, confidence interval; CO, cryptorchidism; JPN, Japan; OR, odds ratio; SNP, single-nucleotide polymorphism.
*$P<0.05$ in Fisher's exact test at genotypic level.
#$P<0.05$ in Cochran–Armitage trend test corrected with Benjamini–Hochberg false discovery rate.
[a]Data missing due to inability to amplify DNA.
[b]*P*-value in Cochran–Armitage trend test at allelic level.

CI = 2.0–6.8; OR = 7.2, 95% CI = 2.3–22.5; and OR = 4.0, 95% CI = 1.9–8.5, respectively). Furthermore, the allele frequencies of these SNPs differed significantly between the HS patients and the controls ($P_{trend}<0.05$).

Polymorphisms and CO risk in the ITA study

The SNPs found to be associated with risk of CO in the ITA study group are shown in Table 4. Heterozygous *rs3757824* (AG) of *AHR* and minor homozygous and heterozygous *rs1020397* (CC and CG, respectively) of *ARNT2* were more frequently found in the 58 CO patients than in the 129 controls (OR = 3.1, 95% CI = 1.6–6.1; OR = 3.4, 95% CI = 1.3–8.9; and OR = 2.8, 95% CI = 1.3–5.8, respectively). The allele frequencies of these SNPs also differed significantly different between CO patients and controls ($P_{trend}<0.05$). None of the SNPs positively associated with CO risk were found to be common to both JPN and ITA populations.

Possible gene–gene interaction in predisposition of CO and HS

Table 5 presents the potential gene–gene interaction in predisposition for CO and HS among the positive SNPs identified in this study using multifactor dimensionality reduction analysis. For all possible interactions among the positively and negatively associated SNPs, the most significant gene–gene interplay were *rs2472680–rs4919686–rs5000770* with a 62.81% prediction accuracy for CO ($P = 0.011$) and *rs2069521–rs2278705* with a 69.98% prediction accuracy for HS ($P = 0.001$) in JPN population. In a combined analysis of JPN and ITA population, a multi-locus association was observed between *rs5000770* and *rs3757824*, which had 65.70% prediction accuracy for CO ($P = 0.055$).

DISCUSSION

This study was initiated to increase our understanding of the potential interaction of EED exposure and genetic factors on the risk of developing MGM. To achieve this aim, we sought to identify polymorphisms in genes involved in EED metabolism that were associated with an increased risk of CO and HS in a case–control study of populations from Japan and Italy.

One of our most interesting results concerned SNP *rs5000770* of *ARNT2*. We observed a significant association at both allelic and genotypic levels between *rs5000770* genotype and the risk of both CO and HS in the JPN study group. Patients with the AA genotype had a significant increase in CO and HS risk compared with those with the

Table 3 Effect of *CYP1A2, ARNT2, CYP17A1* and *NR1I2* polymorphisms on the risk of HS in the JPN study group

			Case (n = 98)		*Control (n = 141)*			
Gene	*SNP*	*Genotype*	*No.*[a]	*%*	*No.*	*%*	*OR (95% CI)*	P_{trend}[b]
CYP1A2	rs2069521	GG	22	22.92	85	60.28	Reference	1.59E-11#
		AA	22	22.92	2	1.42	4.5 (9.3–194.6)*	
		AG	52	54.16	54	38.30	3.7 (2.0–6.8)*	
	rs2069522	AA	70	81.40	128	91.43	Reference	0.0502541
		GG	0	0	0	0		
		AG	16	18.60	12	8.57	2.4 (1.1–5.4)*	
ARNT2	rs2278705	GG	61	62.89	104	74.29	Reference	0.0018348#
		AA	17	17.53	4	2.86	7.2 (2.3–22.5)*	
		AG	19	19.59	32	22.85	1.0 (0.5–1.9)	
	rs5000770	GG	35	35.71	78	55.32	Reference	0.000249#
		AA	27	27.55	15	10.64	4.0 (1.9–8.5)*	
		AG	36	36.74	48	34.04	1.7 (0.9–3.0)	
	rs11072922	GG	64	65.31	82	58.16	Reference	0.632887
		AA	14	14.29	4	2.84	4.5 (1.4–14.3)*	
		AG	20	20.40	55	39.00	0.5 (0.3–0.9)	
CYP17A1	rs17115149	CC	69	78.41	116	82.27	Reference	0.145118
		AA	17	19.32	13	9.22	2.2 (1.0–4.8)*	
		AC	2	2.27	12	8.51	0.3 (0.1–1.3)	
NR1I2	rs2461823	AG	33	36.26	67	47.52	Reference	0.58977
		AA	17	18.68	15	10.64	2.3 (1.0–5.2)*	
		GG	41	45.06	59	41.84	1.4 (0.8–2.5)	

Abbreviations: CI, confidence interval; HS, hypospadias; JPN, Japan; OR, odds ratio; SNP, single-nucleotide polymorphism.
*$P<0.05$ in Fisher's exact test at genotypic level.
#$P<0.05$ in Cochran–Armitage trend test corrected with Benjamini–Hochberg false discovery rate.
[a]Data missing due to inability to amplify DNA.
[b]*P*-value in Cochran–Armitage trend test at allelic level.

Table 4 Effect of AHR and ARNT2 polymorphisms on the risk of CO in the ITA study group

			Case (n = 58)		*Control (n = 129)*			
Gene	*SNP*	*Genotype*	*No.*[a]	*%*	*No.*	*%*	*OR (95% CI)*	P_{trend}[b]
AhR	rs3757824	AA	27	46.55	93	72.09	Reference	0.0029
		GG	4	6.90	6	4.65	2.3 (0.6–8.7)	
		AG	27	46.55	30	23.26	3.1 (1.6–6.1)*	
ARNT2	rs1020397	GG	13	22.41	59	45.74	Reference	0.0039
		CC	12	20.69	16	12.40	3.4 (1.3–8.9)*	
		CG	33	56.90	54	41.86	2.8 (1.3–5.8)*	

Abbreviations: CI, confidence interval; CO, cryptorchidism; ITA, Italian; OR, odds ratio; SNP, single-nucleotide polymorphism.
*$P<0.05$ in Fisher's exact test at genotypic level.
[a]Data missing due to inability to amplify DNA.
[b]*P*-value in Cochran–Armitage trend test at allelic level.

GG genotype. Furthermore, synergistic interactions between *rs5000770* and SNPs in *NR1I2*, *CYP17A1*, *AHR* and *CYP1A2*, which might confer susceptibility to both CO and HS in the JPN study group, were observed in the multifactor dimensionality reduction analysis. ARNT2 is a member of the basic helix–loop–helix Per-ARNT-SIM (bHLH-PAS) family of transcription factors that is involved in the regulation of many physiological pathways, including responses to environmental contaminants and oxygen deprivation, and for biological rhythms, angiogenesis and neuronal development.[23–25] *Arnt2* has pivotal roles in the regulation of early development in zebrafish.[26] *ARNT2* polymorphisms have been linked with the risk of some specific congenital malformations in humans such as cleft palate.[27] However, little is known about the relationship of *ARNT2* polymorphisms and the risk of MGM. Recently, a new concept has been suggested that testicular cancer, CO and some cases of HS and impaired spermatogenesis are symptoms of a single underlying entity that has been named the testicular dysgenesis syndrome.[28,29] This concept proposes the existence of a common underlying cause for the occurrence of these reproductive and developmental diseases, and suggests that adverse

Table 5 Gene–gene interaction models for CO and HS

Disease	*Population*	*SNPs included*[a]	*Best model*	*TBA*	*CVC*	*P-value*
CO	JPN population[b]	SNPs significantly related with CO risk in JPN population (*SNP4, 6, 7*)	SNP7	0.5318	7/10	0.377
			SNP6, 7	0.5999	8/10	0.055
			SNP4, 6, 7	0.6281	10/10	0.011
	Combination of JPN and ITA population[c]	SNPs significantly related with CO risk in JPN (*SNP4, 6, 7*) and ITA population (*SNP1, 5*)	SNP5	0.5093	9/10	0.828
			SNP5, 7	0.657	10/10	0.055
			SNP1, 5, 7	0.5704	10/10	0.055
HS	JPN population[d]	SNPs significantly related with HS risk in JPN population (*SNP2, 3, 7*)	SNP2	0.6958	10/10	0.001
			SNP2, 3	0.6998	9/10	0.001
			SNP2, 3, 7	0.6576	10/10	0.001

Abbreviations: CO, cryptorchidism; CVC, cross-validation consistency; HS, hypospadias; JPN, Japan; ; ITA, Italian; SNP, single-nucleotide polymorphism; TBA, testing balanced accuracy.
P-values are from the sign test.
[a]SNP1: rs1020397 (ARNT2); SNP2: rs2069521 (CYP1A2); SNP3: rs2278705 (ARNT2); SNP4: rs2472680 (NR1I2); SNP5: rs3757824 (AHR); SNP6: rs4919686 (CYP17A1); SNP7: rs5000770 (ARNT2).
[b]$n=236$ (141 controls and 95 CO).
[c]$n=423$ (270 controls and 153 CO).
[d]$n=239$ (141 controls and 98 HS).

environmental factors such as EEDs might exert their etiological impacts under a susceptible genetic background. Our result indicates that variations in *ARNT2* may be one of the possible common causes. One possible interpretation of our findings is that the A allele of *ARNT2* might influence individual responsiveness to EEDs, and increase the risk of CO and HS.

The SNP *rs6163* of *CYP17A1* is a common genetic polymorphism in the JPN population with a minor allele frequency of approximately 0.45.[30,31] In the JPN group studied here, individuals with *rs6163* CC genotype appeared to have an increased risk of CO. However, our statistical analysis suggested that the allele frequency difference between patients and controls for this SNP was only on the borderline of significance ($P_{trend}=0.067$). It has been speculated that *CYP17A1* variants might show differences in transcriptional efficiency and enzyme activity that, in turn, affect estrogen and androgen levels.[32] *CYP17A1* variants have been shown to be associated with increased risk of diseases in which estrogens or androgens have an important role, such as breast cancer and prostate cancer.[33–35] Here we suggest that the *rs6163* genotype might affect androgen homeostasis during fetal life and, thereby, increase the risk of MGM as male sexual differentiation is critically dependent on normal androgen concentrations.[36] At present, there is no information regarding any association between the *rs6163* polymorphism and circulating hormone levels; however, another SNP (*rs743572*), which is also located in the 5′-untranslated region, has been extensively investigated and shown to be related to reduced messenger RNA levels in ovarian cells.[32] In addition, we observed a significant association at both allelic and genotypic levels between the SNP *rs4919686* for *CYP17A1* with risk of CO in the JPN study group. However, this variant is much less common than *rs6163* and has only nine carriers in the control group.

The *NR1I2* gene encodes the orphan nuclear receptor PXR, which has broad specificity and activates expression of *CYP* genes in response to a wide variety of xenobiotics. Following activation through ligand binding, PXR binds to the response element and induces the expression of *CYP3A4*, which has a major role in the hydroxylation of both estrone and estradiol.[16] EEDs, especially those with estrogenic effects, may modulate estrogen levels through PXR signaling. Polymorphisms in genes involved in PXR signaling may modify the adverse effect of EED exposure on estrogen levels. In postmenopausal women, an interaction effect between *NR1I2* gene variants and phytoestrogen exposure has been reported to influence circulating sex hormone levels.[37] Our observation here that the heterozygous *rs247280* genotype AG of *NR1I2* is linked with an increased risk for CO in the JPN study group is consistent with this hypothesis. However, no significant association between *CYP3A4* gene variants and risk of CO was found in this study.

The SNPs *rs3757824* of *AHR* and *rs1020397* of *ARNT2* were associated with an increased risk of CO in the ITA study group but not in the JPN group. However, the interaction between *rs3757824* and another polymorphism of *ARNT2* (*rs5000770*) seems to confer susceptibility to CO in a combined analysis of JPN and ITA population (65.70% prediction accuracy, $P=0.055$). Previous studies have reported that genetic polymorphisms in AHR signaling may affect the induction of CYP1A1 and can be related to the risk of endocrine-related diseases, such as breast cancer.[38] A recent study has found a weak interaction effect between *AHR rs3757824* and environmental risk factors on colorectal neoplasia.[39]

The SNP *rs2069521* of *CYP1A2* was found to be significantly associated at both allelic and genotypic levels with risk of HS in JPN study group. CYP1A2 is one of the major CYP1 enzymes involved in the formation of catechol estrogens, which are known to be estrogenic and are thought to be carcinogenic.[40] One possible explanation for this finding may be due to linkage disequilibrium with other genetic variants. CYP1A2 variants are in linkage disequilibrium with CYP1A1 alleles, which themselves have been previously associated with the risk of infertility and HS.[13] Moreover, CYP1A1 and CYP1A2 share many of the same enzymatic activities and may be under coordinated regulation; placental expression and activity of CYP1A1 seem to be greater than for CYP1A2 and to occur earlier in pregnancy.[41] However, we did not find any positive CYP1A1 variants in this study.

We did not find any genetic polymorphisms in common between the JPN and ITA study groups for risk of CO. Various possible factors may underlie the apparent absence of shared polymorphisms. One possible contributing factor is the low number of cases in our study (95 in the JPN group and 58 in the ITA group). Another factor may be the differences between ethnic groups in allele frequencies. Indeed, a somewhat similar result was found in investigations of the association of *ESR1* polymorphisms and CO risk in these two ethnic groups. In the JPN study group, five SNPs in the 3′ region of the *ESR1* gene (the AGATA allele) were found to be overrepresented in cryptorchid patients in comparison with controls (34.0 versus

21.3%), and homozygosity for this variant was found only in patients with undescended testes.[42] By contrast, in the ITA study population, the AGATA haplotype was found to be associated with a reduced risk of CO.[43] However, a *rs5000770–rs3757824* interaction to susceptibility of CO was observed in the combined analysis of these two study groups. This interesting observation might explain partly the possible genetic effects masked by different gene–gene interaction leading to the controversial results in association studies, although further studies are necessary to confirm our findings in different ethnic groups.

Our study has several potential limitations that should be taken into consideration. First, as the study group sizes were relatively small, then the statistical power for the detection of subtle changes might have been limited. Second, we hypothesized that the impaired function of proteins encoded by susceptibility genes might be caused by genetic polymorphisms, and that such impaired function might increase the risk of development of CO and HS. However, little is known of whether such genetic polymorphisms actually affect protein and/or cell functions. Therefore, further studies are needed to confirm our findings and to explore the possible molecular mechanisms of our observations.

In conclusion, this study suggests that polymorphism of genes involved in the metabolism of EEDs might have a significant role in the risk of development of CO and HS. The genes that were studied function in dioxin binding (*AHR* and *ARNT2*), dioxin induction (*CYP1A2*), estrogen synthesis (*CYP17A1*) and bisphenol A induction (*NR1I2*), suggesting a possible link between EED exposure and the development of MGMs. Inter-individual polymorphic differences might cause variations in sensitivity to the effects of EEDs as a potential cause of MGMs.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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Characterization of DNA methylation errors in patients with imprinting disorders conceived by assisted reproduction technologies

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BACKGROUND: There is an increased incidence of rare imprinting disorders associated with assisted reproduction technologies (ARTs). The identification of epigenetic changes at imprinted loci in ART infants has led to the suggestion that the techniques themselves may predispose embryos to acquire imprinting errors and diseases. However, it is still unknown at what point(s) these imprinting errors arise, or the risk factors.

METHODS: In 2009 we conducted a Japanese nationwide epidemiological study of four well-known imprinting diseases to determine any association with ART. Using bisulfite sequencing, we examine the DNA methylation status of 22 gametic differentially methylated regions (gDMRs) located within the known imprinted loci in patients with Beckwith-Wiedemann syndrome (BWS, $n = 1$) and also Silver-Russell syndrome (SRS, $n = 5$) born after ART, and compared these with patients conceived naturally.

RESULTS: We found a 10-fold increased frequency of BWS and SRS associated with ART. The majority of ART cases showed aberrant DNA methylation patterns at multiple imprinted loci both maternal and paternal gDMRs (5/6), with both hyper- and hypomethylation events (5/6) and also mosaic methylation errors (5/6). Although our study may have been limited by a small sample number, the fact that many of the changes were mosaic suggested that they occurred after fertilization. In contrast, few of the patients who were conceived naturally exhibited a similar pattern of mosaic alterations. The differences in methylation patterns between the patients who were conceived naturally or after ART did not manifest due to the differences in the disease phenotypes in these imprinting disorders.

CONCLUSION: A possible association between ART and BWS/SRS was found, and we observed a more widespread disruption of genomic imprints after ART. The increased frequency of imprinting disorders after ART is perhaps not surprising given the major epigenetic events that take place during early development at a time when the epigenome is most vulnerable.

Key words: assisted reproduction technologies / genomic imprinting / DNA methylation / gametic differentially methylated regions / genomic imprinting disorders

Introduction

Human assisted reproduction technologies (ARTs) are used in the treatment of infertility and involve the manipulation of eggs and/or sperm in the laboratory. Several recent studies have identified an increased incidence of some normally very rare imprinting disorders after ART, including Beckwith-Wiedemann syndrome (BWS: ONIM 130650), Angelman syndrome (AS: ONIM 105830) and Silver-Russell syndrome (SRS: OMIM 180860) but not Prader-Willi syndrome (PWS: OMIM 176270; DeBaun *et al.*, 2003; Gosden *et al.*, 2003; Svensson *et al.*, 2005). Additionally, there are several reports suggesting that epigenetic alterations (epimutations) at imprinted loci occur during the *in vitro* manipulation of the gametes, with both IVF and ICSI approaches implicated (Cox *et al.*, 2002; DeBaun *et al.*, 2003; Gicquel *et al.*, 2003; Maher *et al.*, 2003; Moll *et al.*, 2003; Orstavik *et al.*, 2003; Ludwig *et al.*, 2005; Rossignol *et al.*, 2006; Bowdin *et al.*, 2007; Kagami *et al.*, 2007). However, some studies do not support a link between ART and imprinting disorders (Lidegaard *et al.*, 2005; Doornbos *et al.*, 2007).

Epigenetic marks laid down in the male or female germ lines, and which are inherited by the embryos, establish the imprinted expression of a set of developmentally important genes (Surani, 1998). Because imprinted genes are regulated by these gametic epigenetic marks, and by further epigenetic modifications in the somatic cell, they are particularly vulnerable to environmentally induced mutation. One of the best studied epigenetic marks is DNA methylation. DNA methylation is established in either the maternal or paternal germline at discrete genomic loci. This methylation is preserved in the fertilized embryo to generate differentially methylated regions (DMRs) which then signal to nearby genes to establish domains of imprinted chromatin by mechanisms that are not fully understood (John and Lefebvre, 2011). These germline or gametic DMRs (gDMRs) can orchestrate the monoallelic expression of genes over megabases of DNA (Tomizawa *et al.*, 2011) and are reset with every reproductive cycle (Lucifero *et al.*, 2002; Obata and Kono, 2002).

The increased frequency of epimutation(s) at imprinted loci in ART infants has led to the suggestion that ART procedures may induce imprinting error(s). However, these studies are confounded because ART populations are, by their very nature, different from populations who were conceived without the use of ART, with a low fertility rate, an increased frequency of reproductive loss and usually of advanced age, all of which are associated with increased occurrence of fetal and neonatal abnormalities. Furthermore, it is difficult to determine the causality of imprinting errors in any specific abnormality reported after ART. Both IVF and ICSI appear to be associated with an increased relative risk of imprinting disorders (Savage *et al.*, 2011). These procedures are often undertaken for unexpected infertility and require ovarian stimulation, oocyte collection and *in vitro* culture before the embryos are implanted. It has been suggested that infertility and any resulting ovarian stimulation may predispose to epigenetic errors (Sato *et al.*, 2007). Animal studies suggest that *in vitro* embryo culture may be associated with epigenetic alterations. In particular, the large offspring syndrome in cattle undergoing ART is associated with the loss of maternal allele methylation at insulin-like growth factor 2 receptor (*IGF2R*) gDMR (Young *et al.*, 2001) and has phenotypic similarity to BWS. It is still unknown when these imprinting errors arise and what factors predispose to epigenetic changes. Previously, Chang *et al.* (2005) reported no phenotypic differences between BWS patients who were conceived after ART and naturally. However, Lim *et al.* (2009) reported that patients who were conceived after ART had a significantly lower frequency of exomphalos and higher risk of non-Wilms tumor neoplasia. Phenotypic differences between patients who were conceived after ART and naturally are largely unreported, while any changes to phenotype may be altered by the frequency and the degree of epimutations. Studies revealed that some patients with BWS born after ART presented with epimutations that were not restricted to the 11p15 region (Rossignol *et al.*, 2006; Bliek *et al.*, 2009; Lim *et al.*, 2009). Further analysis of abnormal methylation patterns in imprinting disorders may provide clues as to the cause of disease and identify the ART-related risk factor(s).

To address these questions in this study, we engaged in a nationwide epidemiological study of the Japanese population to determine the frequency of four imprinting disorders after natural conception and after ART. We then analyzed the DNA methylation status of 22 gDMRs in BWS and SRS patients conceived by the two routes. Finally, we compared the abnormal methylation patterns and the phenotypes reported for both sets of patients. As a result we found that both BWS and SRS were more frequent after ART and that ART patients exhibited a higher frequency of aberrant DNA methylation patterns at multiple loci with, in some cases, mosaic methylation errors.

Materials and Methods

Nationwide investigation of imprinting disorders

The protocol was established by the Research Committee on the Epidemiology of Intractable Diseases. The protocol consisted of a two-stage postal survey. The first-stage survey was used to estimate the number of individuals with any of the four imprinting diseases: BWS, SRS, PWS and AS. The second-stage survey was used to identify the clinico-epidemiological features of these syndromes.

In the first-stage survey, the pediatric departments of all hospitals were identified based on a listing of hospitals, as at 2008, supplied by the R&D Co. Ltd (Nagoya, Japan). Hospitals were classified into seven categories according to the type of institution and the number of hospital beds. The survey was mailed to a total of 3158 departments in October 2009 with letters of request for participation in recording these diseases. A simple questionnaire was used to ask about the number of patients with any of the four imprinting disorders. Diagnosis was determined by karyotype analyses, genetic analyses and clinical phenotypes by their clinical doctors. In December 2009, a second request was sent to departments that had not responded to the earlier deadline (at the end of November 2009). Following the first-stage survey, we sent acknowledgement letters to departments that had responded.

The second questionnaires were forwarded to the departments that had reported patients with the imprinting disorders on the first questionnaires. Detailed clinical information for the patients with these imprinting disorders was collected, including the age, gender, growth and development pattern, the methods of the diagnosis, the presence of infertility treatment and the methods of ART where applicable. Duplicate results were excluded using the information regarding the patient's age and gender where available. The study was approved by the Ethics Committee of Tohoku University School of Medicine.