and stomach cancers [30,32–34]. Several MMPs have been implicated in ECM degradation associated with tumor growth and angiogenesis, which is required for a cancer cell to invade a nearby blood vessel (intravasation) and then to extravasate at a distant location and invade the distant tissue in order to seed a new metastatic site [35].

To our knowledge, there have not been any reports of human congenital genital disorders associated with MMP11. However, it has been reported that MMPs play a critical role in cell fate and behavior during many developmental processes [31,36]. Both genetic analysis using transgenic mice and pharmacogenetic studies with chemical inhibitors have elucidated that loss of function of MMPs, in particular MMP11, might induce dysregulation in cell migration and apoptosis during tissue remodeling or branching of mammary epithelial cells [37,38]. A more recent study in the model insect, *Tribolium*, explored MMP functions *in vivo* and found that knockdown of MMPs using genetic interference resulted in malformation in tracheal and gut development during beetle embryogenesis and pupal morphogenesis [39]. It is known that epithelial seam formation and remodeling during urethral formation play important roles in the etiology of HS. The urethral abnormalities seen in HS can be viewed as a failure of epithelial cell adhesion [40]. Therefore, we hypothesized that downregulation of MMP11 expression might decrease cellular adhesion in the developing male urethra and ventral penile skin, which might result in the abortive penile development seen in HS.

To further confirm this hypothesis, we compared the expression levels of MMP11 in hFFCs derived from child HS and CO patients ($n = 23$ and 11, respectively). In 2001, Skakkebaek and his colleagues proposed a concept of TDS: impaired development of fetal testes could lead to increased risks of CO, HS, decreased spermatogenesis or testicular cancer [2]. However, they have recently changed their opinion and now suggest that HS is only marginally associated with TDS [3]. Although much remains to be determined, it is likely that the molecular etiology of HS and CO is different. CO is the absence of one or both testes from the scrotum and is the most common congenital abnormality in boys with a reported prevalence at birth of approximately 2–9%, according to registry data [41]. Impaired descent of the testes is thought to be fetal in origin, and if the *in utero* development of the testicles is impaired then their production of insulin-like factor 3 and especially testosterone may be reduced, which may lead to some degree of CO [3,42]. However, it is likely that isolated HS may have a different etiological mechanism, including a congenital developmental problem restricted to the penis [43]. Rey *et al.* found that most boys (85%) with isolated HS had, in general, normal testicular endocrinology in contrast to those with HS combined with other genital abnormalities [44]. In this study, only child HS and CO patients without other genital malformations of syndromes were recruited. Therefore, hFFCs derived from foreskin tissues of child CO patients might be viewed as the control group in this study. We found that MMP11 expression in the HS group was significantly lower than in the CO group (0.25-fold, $P = 0.0027$) (Figure 4). This result is in accordance with our hypothesis that downregulation of MMP11 expression might be related with the pathology of HS. Although the urethral tissue was not directly examined, it is possible that there is also a potential effect of MMP11 on urethral development.

In summary, the present study examined targets of low-dose BPA exposure and transcriptome differences in response to BPA, E2 and TCDD in hFFCs derived from child HS patients using DNA microarray analysis. Of particular interest, the expression of MMP11 was found to be downregulated by BPA in a dose-dependent manner. Furthermore, we also found that MMP11 expression in the HS group was significantly lower than in the CO

group. Our findings suggested that the involvement of BPA in the development of HS might relate to downregulation of MMP11 expression. Further study of the novel target genes identified in this study during genital tubercle development might increase our knowledge of the molecular basis of the effects of BPA on human reproductive health.

Supporting Information

Figure S1 Red indicates upregulated genes, green indicates downregulated genes, and white indicates genes that were not annotated in this array but form part of this network. The bottom numbers indicate the fold changes induced by BPA and the top numbers is the P-values between DMSO control group and BPA treated group. (*A*) "Cellular Growth and Proliferation, Hematological System Development and Function, Cellular Development" network; (*B*) "Cellular Assembly and Organization, Cellular Function and Maintenance, Cell Cycle" network.
(DOCX)

Figure S2 Red indicates upregulated genes, green indicates downregulated genes, and white indicates genes that were not annotated in this array but form part of this network. The bottom numbers indicate the fold changes induced by E2 and the top numbers is the P-values between DMSO control group and E2 treated group. (*A*) "Cellular Growth and Proliferation, Skeletal and Muscular System Development and Function, Cell Cycle" network; (*B*) "DNA Replication, Recombination, and Repair, Gene Expression, Cellular Assembly and Organization" network; (*C*) "Cellular Assembly and Organization, Cellular Function and Maintenance, Protein Synthesis" network; (*D*) "Gene Expression, Cell Cycle, Cell-To-Cell Signaling and Interaction" network; (*E*) "DNA Replication, Recombination, and Repair, Nucleic Acid Metabolism, Small Molecule Biochemistry" network.
(DOCX)

Figure S3 Red indicates upregulated genes, green indicates downregulated genes, and white indicates genes that were not annotated in this array but form part of this network. The bottom numbers indicate the fold changes induced by TCDD and the top numbers is the P-values between DMSO control group and TCDD treated group. (*A*) "Post-Translational Modification, Genetic Disorder, Hematological Disease" network; (*B*) "Cell Cycle, Cellular Assembly and Organization, DNA Replication, Recombination, and Repair" network; (*C*) "Cellular Assembly and Organization, DNA Replication, Recombination, and Repair, Decreased Levels of Albumin" network; (*D*) "DNA Replication, Recombination, and Repair, Energy Production, Nucleic Acid Metabolism" network; (*E*) "DNA Replication, Recombination, and Repair, Cell Cycle, Cellular Assembly and Organization" network.
(DOCX)

Figure S4 "Pathways in cancer" of KEGG was mapped with genes significantly differentially expressed in response to BPA (*A*), E2 (*B*) and TCDD (*C*).
(DOCX)

Table S1 Comparison of the gene expression profiles of hFFCs in response to BPA, E2 and TCDD.
(DOCX)

Table S2 KEGG pathways affected by BPA, E2 and TCDD identified by Pathway Express.
(DOCX)

Acknowledgments

Author Contributions

Conceived and designed the experiments: TF J. Yoshinaga J. Yonemoto MF TO HS. Performed the experiments: XYQ HZ HA QZ. Analyzed the data: XYQ. Contributed reagents/materials/analysis tools: YK K. Mizuno KU K. Muroya MM KK YH MF TO. Wrote the paper: XYQ HS.

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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 at 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 −1.15 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 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 regulatory mutations of *SOX9*. © 2012 Wiley Periodicals, Inc.

Key words: campomelic dysplasia; deletion; inversion; enhancer

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.
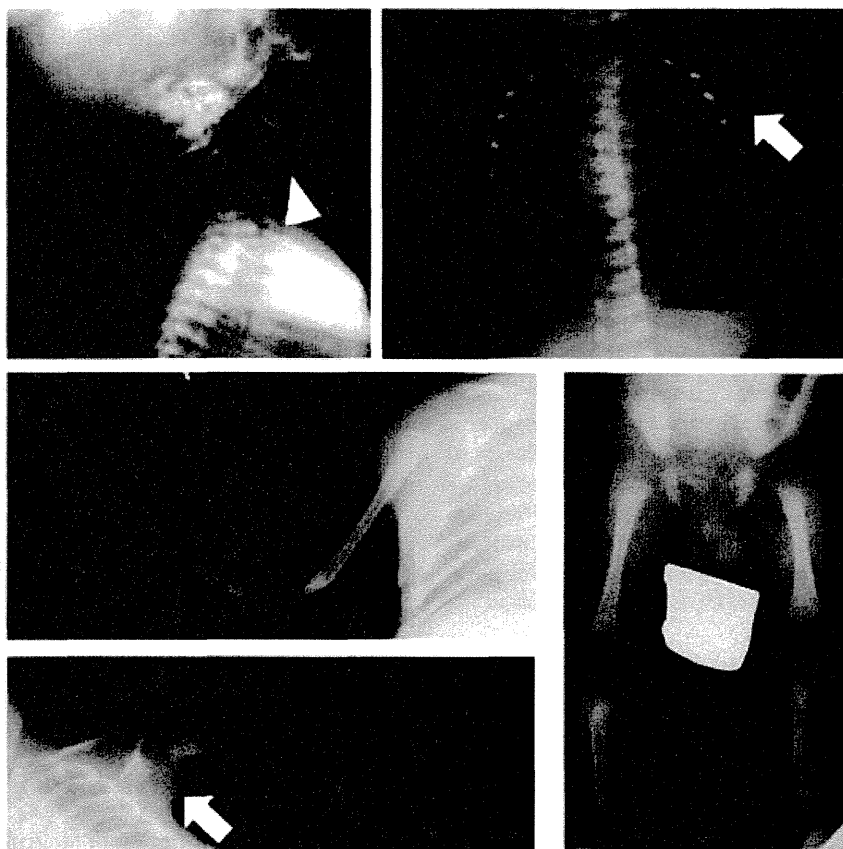

FIG. 1. Roentgenograms of the patient at 2 weeks of age. Mild hypoplasia of left scapula (white arrows) and micrognathia (a white arrowhead) are indicated.

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
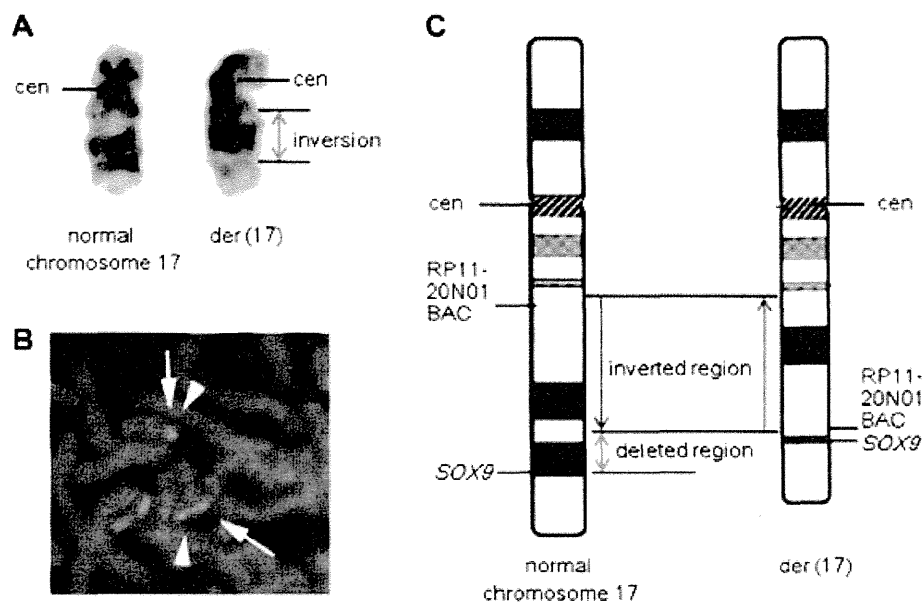
FIG. 2. Chromosomal banding and FISH analysis. A: High-resolution chromosomal banding indicating the presence of a deletion and an inversion on the long arm of chromosome 17. cen, centromere. B: Representative results of FISH analysis. The arrowheads denote RP11-84E24-BAC containing *SOX9* (green signals); 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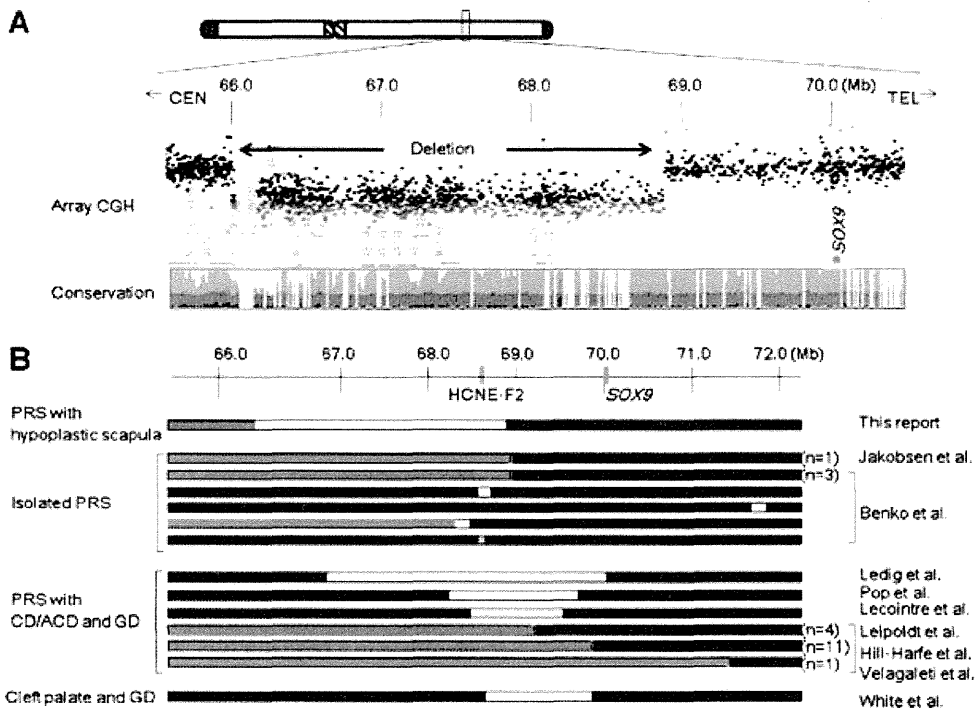 the Human Genome (February 2009, hg 19; NCBI Build 37). B: Schematic representation of genomic lesions and clinical features of present case and previously reported patients [Pop et al., 2004; Hill-Harfe et al., 2005; Velagaleti et al., 2005; Jakobsen et al., 2007; Leipoldt et al., 2007; Benko et al., 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

Haplotype analysis of *ESR2* in Japanese patients with spermatogenic failure

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The prevalence of spermatogenic failure (SF) has gradually increased during the past few decades at least in several countries. Although multiple factors would be involved in this phenomenon, one important factor would be excessive estrogen effects via estrogen receptors (ERs). Thus, we performed haplotype analysis of *ESR2* encoding ERβ in 125 Japanese SF patients and 119 age-matched control males, using single nucleotide polymorphisms (SNPs) 1–9 that are widely distributed on the ~120-kb genomic sequence of *ESR2*. Consequently, a linkage disequilibrium (LD) block was detected in an ~60-kb region encompassing SNPs 2–7 in both groups, and four major estimated haplotypes were identified within the LD block. Furthermore, the most prevalent 'TGTAGA' haplotype was found to be significantly associated with SF, with the *P*-value obtained by the Cochran–Armitage trend test (0.0029) being lower than that obtained by a 100 000-times permutation test (0.0038) to cope with the problem of multiple comparisons. The results, in conjunction with our previous data indicating lack of a susceptibility factor on *ESR1* encoding ERα, imply that the specific 'TGTAGA' haplotype of *ESR2* raises the susceptibility to the development of SF.
Journal of Human Genetics (2012) **57**, 449–452; doi:10.1038/jhg.2012.53; published online 24 May 2012

Keywords: environmental endocrine disruptors; *ESR2*; estrogenic effects; haplotype analysis; spermatogenic failure; susceptibility

INTRODUCTION

Recent studies have indicated a gradual increase in the prevalence of male genial and reproductive abnormalities during the past few decades at least in several countries.[1] Skakkebaek *et al.*[2] have coined a term 'testicular dysgenesis syndrome' for this phenomenon. As such deterioration of male genital and reproductive health is also observed in many wildlife species,[1,3] it is likely that such adverse changes in males are inter-related events shared in common by the human and the wildlife species.[1,3] In this regard, environmental endocrine disruptors (EEDs) appear to constitute the major factor for this phenomenon, because EEDs are widely spread in the world.[1,3] In particular, exposure to estrogenic EEDs are known to affect male genital and reproductive health.[1,3–5]

The effects of EEDs would primarily be determined by the genetic susceptibility, together with the dosage of exposed EEDs, character of exposed EEDs (for example, estrogenic, anti-androgenic and so on), and the developmental stage of the individuals at the time of EED exposure.[1,3] In this regard, it is known that estrogenic EEDs can bind to both estrogen receptor (ER)α encoded by *ESR1* and ERβ encoded by *ESR2* with low but variable degrees of affinities.[3] Thus, it is likely that genetic susceptibility to estrogenic EEDs is primarily constituted by genetic variations in *ESR1* and *ESR2*.[1,3]

To examine this possibility, we have previously performed haplotype analysis of *ESR1* in Japanese male patients with genial and reproductive abnormalities as well as in control males, using 15 single nucleotide polymorphisms (SNPs 1–15) that are widely distributed throughout the >300-kb genomic sequence of *ESR1*.[6,7] Consequently, we identified an ~50-kb linkage disequilibrium (LD) block spanning SNPs 10–14 in the 3′ region of *ESR1*, and found that homozygosity of a specific 'AGATA' haplotype within the LD block was strongly associated with cryptorchidism (*P* = 0.0040; odds ratio (OR) = 7.55) and hypospadias (*P* = 0.000057; OR = 13.75)[6,7] (and our unpublished updated observation). This finding provides strong evidence that homozygosity of the specific *ESR1* haplotype raises the susceptibility to the development of male genital abnormalities. In this context, we speculate that this effect via the specific *ESR1* haplotype is mediated by EEDs, although there is no direct evidence yet. Indeed, as *ESR1* is expressed in Leydig cells producing testosterone and insulin-like 3,[5,8] it is likely that the specific *ESR1* haplotype primarily enhances estrogenic effects in Leydig cells, compromising their hormonal production capacity.

However, no significant association was found between the specific 'AGATA' haplotype of *ESR1* and spermatogenic failure (SF).[7] In this context, as *ESR2* is clearly expressed in various developmental stages of male germ cells,[5] it may be possible that the deleterious effects of estrogenic EEDs on spermatogenesis may primarily be mediated by ERβ. Thus, we carried out haplotype analysis of *ESR2* in Japanese patients with SF.

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Received 28 November 2011; revised 25 April 2012; accepted 28 April 2012; published online 24 May 2012

MATERIALS AND METHODS

Subjects

We studied 125 SF patients aged 32–52 years (median 41.0 years), including 80 SF patients utilized in the previous *ESR1* haplotype analysis.[7] The selection criteria included: (1) azoospermia or severe oligozoospermia (<5 million sperms per ml) demonstrated by two consecutive analyses of semen obtained after 4–7 days of abstinence; (2) lack of extragenital anomalies such as cryptorchidism and hypospadias; (3) hypergonadotropic hypogonadism indicative of primary testicular dysfunction; (4) no seminal tract obstruction, varicocele, or retrograde ejaculation; (5) a 46,XY karyotype with no demonstrable structural or numerical abnormality after examining ⩾30 lymphocytes; (6) absence of a Y chromosomal microdeletion after examining 36 loci from *SRY* to *DYZ1*, including multiple Yq loci in the azoospermia factor regions (AZFa, b, c) such as *RBMY* and *DAZ*;[9] (7) no significant expansion of CAG repeat length at exon 1 of *AR* that is known to raise the susceptibility to male reproductive abnormalities;[10] and (8) lack of a disease episode that could affect fertility such as mumps orchiditis. For controls, 119 control adult males with proven fertility aged 24–50 years (median 35.5 year) were similarly analyzed with permission. The ages were similar between the SF patients and control males (Mann–Whitney's *U*-test). All the SF patients and control males were Japanese living in the Tokyo urban area; they were free from particular residential environments such as the vicinity of chemical factories or farms, from specific dietary habits deviated to vegetables or animal/fish proteins, and from intake of drugs with hormonal effects.

SNP analysis

This study was approved by the Institutional Review Board Committees of the authors, and informed consent was obtained from each subject. We examined nine SNPs (SNPs 1–9) that were associated with high minor allele frequencies in the Japanese population (20.3–39.5%) (the NCBI Short Genetic Variations Database (dbSNP); http://www.ncbi.nlm.nih.gov/snp/) and were widely distributed on the ∼120-kb *ESR2* genomic DNA sequence including an apparent LD block encompassing exons 1–6 identified in various populations (the International HapMap Project Database; http://hapmap.ncbi.nlm.nih.gov/) (Figure 1a). Genotyping was performed by the 5′ nuclease assay on an ABI PRISM 7000 Sequence Detection System (Life Technologies, Carlsbad, CA, USA),[11] using leukocyte genomic DNA of each subject.

Pearson's χ²-test with one degree of freedom was applied to test whether the genotyping data are in the Hardy–Weinberg equilibrium. Statistical significance of the differences in allele and genotype frequencies was analyzed by

Pearson's χ²-test, using R environment for statistical computing (http://www.r-project.org/).

Haplotype analysis

Although haplotypes are usually not observed, the haplotypes present in a subject and the frequencies of the haplotypes in a population can be inferred using genotype data at separate loci.[32] In this regard, the degree of LD can be expressed as the pairwise |*D*′| value (the absolute value for the disequilibrium parameter) that ranges from 0 (complete absence of LD) to 1.0 (complete presence of LD),[13] and a chromosomal region associated with high |*D*′| values between different loci is defined as a haplotype or an LD block.[14] In this study, haplotype inference was performed by the maximum-likelihood method using expectation maximization algorithm implemented in the software LDSUPPORT.[15,16] The pairwise |*D*′| values were estimated by the method of Terwilliger and Ott,[12] and a haplotype block was determined by the method of Zhu *et al.*[17] using the software developed by Kamatani *et al.*[18]

The difference in the frequencies of haplotypes between the SF patients and the control males was examined using the estimated population haplotype frequencies by Pearson's χ²-test, and the OR and the 95% confidence interval (CI) were calculated using the R environment. The association between SF phenotype and estimated haplotypes was tested using PENHAPLO software in a dominant mode (comparison of the frequencies of subjects with one risk haplotype between cases and controls) and in a recessive mode (comparison of the frequencies of subjects with two risk haplotypes between cases and controls).[19] Furthermore, the association between SF phenotype and estimated haplotypes was also examined in a dosage-dependent mode (comparison of the frequencies of subjects with zero, one, and two risk haplotypes between cases and controls) by the Cochran–Armitage trend test,[20,21] using the R environment. To cope with the problem of multiple comparisons, the significant level was determined by a 100 000-times permutation test.[22]

RESULTS

SNP analysis

The results of SNP analysis are summarized in Table 1. Minor allele frequencies of the 9 SNPs were 20.4–46.8% in the SF patients and 27.7–37.3% in control males. The genotype frequencies of SNPs 1–9 were in accord with the Hardy–Weinberg equilibrium. Low *P*-values (<0.05) were identified for the differences in the allele and genotype frequencies of SNPs 1, 4, and 5, with stronger association being identified for the
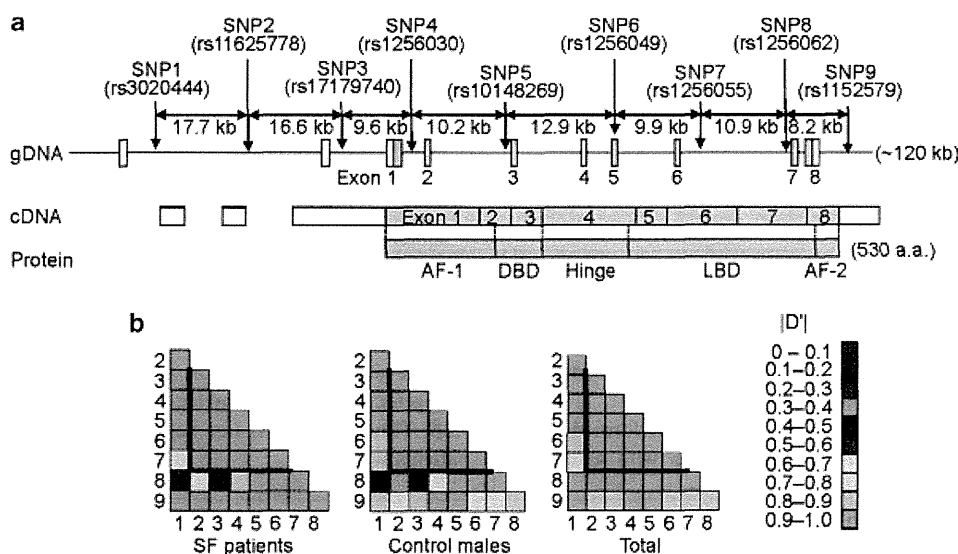
Figure 1 Schematic representation of *ESR2* and its LD maps. (a) Physical positions of *ESR2* SNPs 1–9 examined in the present study. The gray and the white boxes represent coding and untranslated regions, respectively. AF-1, activation function 1 (ligand independent); AF-2: activation function 2 (ligand dependent); DBD, DNA-binding domain; LBD, ligand-binding domain. (b) Pairwise LD maps. |*D*′|: an absolute value for the disequilibrium parameter.

Table 1 Summary of SNP analysis

		Genotyping data			Statistical data		
	Genotype	SF	CM		P-value	OR	95% CI
SNP1	TT	78	58	T vs C	0.028	1.59	1.05–2.42
rs3020444	TC	43	53	TT vs TC + CC	0.032	0.57	0.34–0.95
	CC	4	8	TT + TC vs CC	0.20	2.18	0.64–7.44
SNP2	TT	68	63	T vs C	0.74	1.07	0.72–1.56
rs11625778	TC	48	46	TT vs TC + CC	0.82	0.94	0.57–1.56
	CC	9	10	TT + TC vs CC	0.73	1.18	0.46–3.02
SNP3	GG	77	59	G vs A	0.059	1.49	0.98–2.25
rs17179740	AG	43	52	GG vs AG + AA	0.059	0.61	0.37–1.02
	AA	5	8	GG + AG vs AA	0.34	1.73	0.55–5.45
SNP4	CC	36	55	C vs T	0.0022	1.77	1.23–2.56
rs1256030	CT	61	49	CC vs CT + TT	0.0049	2.13	1.25–3.61
	TT	28	15	CC + CT vs TT	0.045	0.500	0.25–0.99
SNP5	GG	36	55	G vs A	0.0022	1.77	1.23–2.56
rs10148269	AG	61	49	GG vs AG + AA	0.0049	2.13	1.25–3.61
	AA	28	15	GG + AG vs AA	0.045	0.500	0.25–0.99
SNP6	GG	68	64	G vs A	0.74	1.07	0.72–1.60
rs1256049	GA	49	45	GG vs GA + AA	0.92	0.98	0.59–1.61
	AA	8	10	GG + GA vs AA	0.55	1.34	0.51–3.52
SNP7	AA	68	64	A vs G	0.74	1.07	0.72–1.60
rs1256055	AG	49	45	AA vs AG + GG	0.92	0.98	0.59–1.61
	GG	8	10	AA + AG vs GG	0.55	1.34	0.51–3.52
SNP8	AA	59	47	A vs G	0.21	1.27	0.87–1.85
rs1256062	AG	54	57	AA vs AG + GG	0.22	0.73	0.44–1.21
	GG	12	15	AA + AG vs GG	0.45	1.36	0.61–3.04
SNP9	GG	40	45	G vs A	0.12	1.34	0.93–1.92
rs1162579	GA	59	59	GG vs GA + AA	0.34	1.29	0.76–2.19
	AA	26	15	GG + GA vs AA	0.087	0.55	0.28–1.10

Abbreviations: CI, confidence interval; OR, odds ratio; SNP, single nucleotide polymorphism. NCBI rs no. is given for each SNP. SF, 125 patients with spermatogenic failure; CM, 119 control males.

Table 2 Summary of haplotype analysis (SNPs 2–7)

Estimated haplotype	TGTAGA	TACGGA	CGCGAG	TGCGGA
SF (n = 125)	46.4%	21.2%	26.0%	6.0%
CM (n = 119)	32.7%	28.1%	27.3%	11.0%
Comparison of estimated haplotype frequency				
P-value	0.0028	0.096	0.82	0.070
OR	1.77	0.69	0.94	0.52
95% CI	1.21–2.61	0.44–1.06	0.61–1.43	0.25–1.05
Association of estimated haplotype with phenotype				
Dominant mode				
P-value	0.0063	0.078	0.92	0.031
OR	2.08	0.63	0.98	0.46
95% CI	1.23–3.54	0.38–1.05	0.59–1.62	0.22–0.93
Recessive mode				
P-value	0.026	0.34	0.55	0.97
OR	2.16	0.58	0.75	0.95
95% CI	1.09–4.46	0.17–1.79	0.28–1.96	0.037–24.2
Cochran–Armitage's trend test				
P-value	0.0029	0.071	0.75	0.056
For one haplotype				
OR	1.75	0.67	0.94	0.52
95% CI	1.21–2.52	0.44–1.03	0.63–1.39	0.27–1.02
For two haplotypes				
OR	3.06	0.45	0.88	0.27
95% CI	1.46–6.35	0.19–1.06	0.39–1.93	0.07–1.04

Abbreviations: CI, confidence interval; OR, odds ratio; SNP, single nucleotide polymorphism. SF, 125 patients with spermatogenic failure; CM, 119 control males.

allele rather than the genotype frequencies. In particular, the *P*-values for allele frequencies of SNPs 4 and 5 were markedly low.

Haplotype analysis

The LD map is shown in Figure 1b, and the results of haplotype analysis are summarized in Table 2. An ~60-kb LD block spanning SNPs 2–7 was identified in both the SF patients and control males, with the |*D'*| value being >0.9 for all the pairs of SNPs 2–7. Within the LD block, four major estimated haplotypes were identified, together with three additional minor haplotypes ('CGTAGA' haplotype in a single control male, and 'TATAGA' and 'CGCGGA' haplotypes in single SF patients). Notably, the frequency of the most prevalent 'TGTAGA' haplotype was significantly higher in the SF patients than in the control males. Furthermore, the 'TGTAGA' haplotype was significantly associated with SF phenotype, with the *P*-value obtained by the Cochran–Armitage trend test (0.0029) being lower than the permutation *P*-value (0.0038). In addition, of the four major haplotypes, the 'TGTAGA' haplotype alone contained the 'T' allele in SNP 4 and the 'A' allele in SNP 5, whereas these two alleles were also identified in two of the three minor haplotypes.

DISCUSSION

The present study revealed the presence of an ~60-kb LD block encompassing SNPs 2–7 of *ESR2* in both the SF patients and control males. In this regard, the allele frequencies obtained in the control males are comparable to those registered in the JSNP Database, and the LD

block identified in this study is similar to that reported in the International HapMap Project. These findings argue for the accuracy of our data.

Of the four major estimated haplotypes within the LD block, the 'TGTAGA' haplotype was significantly associated with SF. Indeed, the *P*-value obtained by the Cochran–Armitage trend test was below the permutation *P*-value. Furthermore, comparison of the *P*-values obtained from the three types of analyses for the association between SF phenotype and estimated haplotypes implies that the specific 'TGTAGA' haplotype compromises spermatogenesis in a dosage-dependent manner rather than in a simple dominant or recessive manner. In this regard, as the 'T' allele of SNP 4 and the 'A' allele of SNP 5 are almost exclusively present in the 'TGTAGA' haplotype, genotyping of SNPs 4 and 5 can be utilized for the screening of the 'TGTAGA' haplotype.

For *ESR2*, previous studies have suggested an association between SF and an *RsaI* SNP on exon 5 that does not result in amino acid change (SNP 6 in this study) in Scandinavian and Iranian populations (*P*-value: 0.01 and 0.012, respectively).[23,24] In such studies, as the frequency of AG genotype relative to GG genotype was higher in SF patients than in control males (AA genotype was extremely rare), this would imply that the 'A' allele of SNP 6 is regarded as a marker for a hidden true susceptibility factor(s) that is probably in an LD status with the 'A' allele of SNP 6. By contrast, the present study showed no association of SF with SNP 6 and rather suggests a dosage effect of the specific haplotype harboring the 'G' allele of SNP 6. Thus, the present data are apparently inconsistent with the previous studies. It might be possible, however, that the true susceptibility factor(s) is linked with the specific 'TGTAGA' haplotype in the Japanese population and resides on a different pattern of haplotype carrying the 'A' allele of SNP 6 in Scandinavian and Iranian populations, because of a recombination between the true susceptibility factor(s) and SNP 6 in either of the ethnic groups. In addition, there might be population-

specific susceptibility factors, and false positive results might be obtained in association studies with multiple comparisons. This matter awaits further studies.

One may argue that although the present study indicates an association of the specific *ESR2* haplotype with SF, there is no direct evidence for estrogenic EEDs being involved in the development of SF. Indeed, it may be possible that an interaction between the specific *ESR2* haplotype and endogenous estrogens rather than estrogenic EEDs actually underlie the development of SF. However, estrogenic effects of EEDs are known to be primarily mediated by ER.[1,3] In addition, as all the SF patients and the control males examined in this study were apparently free from high exposure to EEDs, the amount of exposed EEDs would be similar between the two groups of subjects. Thus, although further studies such as the investigation of subjects with a high risk of EEDs exposure (for example, workers at chemical factories) are necessary, our results would suggest that the specific *ESR2* haplotype constitutes a susceptibility factor for the development of SF in response to estrogenic EEDs in males who live in an ordinary condition with no high risk of EEDs exposure.

Several points should be made with respect to the present study. First, the number of subjects analyzed remains rather small. Second, the true susceptibility factor(s) on the specific haplotype remains to be identified, although the specific 'TGTAGA' haplotype would facilitate the development of SF by enhancing the ERβ signaling. Third, it remains possible that another susceptibility factor(s) is present on *ESR2*. In particular, as only a few of SNPs were examined in non-LD block regions, a different susceptibility factor(s) may be present on the non-LD block regions of *ESR2*. Fourth, several patients may have some unidentified pathologic cause(s) for SF such as single gene disorders. Fifth, there may be some unknown minor genetic and environmental differences between the patients and the control males. In this context, as SF becomes discernible in adulthood, such minor differences, if they exist, may exert unfavorable influences on spermatogenic function for a long time, leading to SF. This may explain why the OR obtained in this study remained low, in contrast to the high ORs identified in cryptorchidism (7.55) and hypospadias (13.75)[6,7] (and our unpublished updated observation) which develop during the fetal life. Sixth, although it is known that EEDs also exert anti-androgenic effects and influence aromatization,[25,26] these have not been examined in this study. Lastly, it remains to be determined whether similar results can be reproduced in other case–control studies.

Despite the above caveats, this study provides a useful clue to clarify the genetic susceptibility to estrogenic EEDs. In summary, we propose that the specific *ESR2* haplotype raises the susceptibility to the development of SF in response to estrogenic EEDs. Further studies including similar haplotype analyses in different ethnic groups from both developed and developing countries will serve to clarify the relative importance of the dosage of exposed EEDs and the genetic heterogeneity obtained in the process of natural human selection, in the presumably EEDs-related phenomenon such as SF.

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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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.
Journal of Human Genetics (2012) 57, 434–441; doi:10.1038/jhg.2012.48; published online 31 May 2012

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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Received 25 November 2011; revised 26 March 2012; accepted 18 April 2012; published online 31 May 2012

(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%
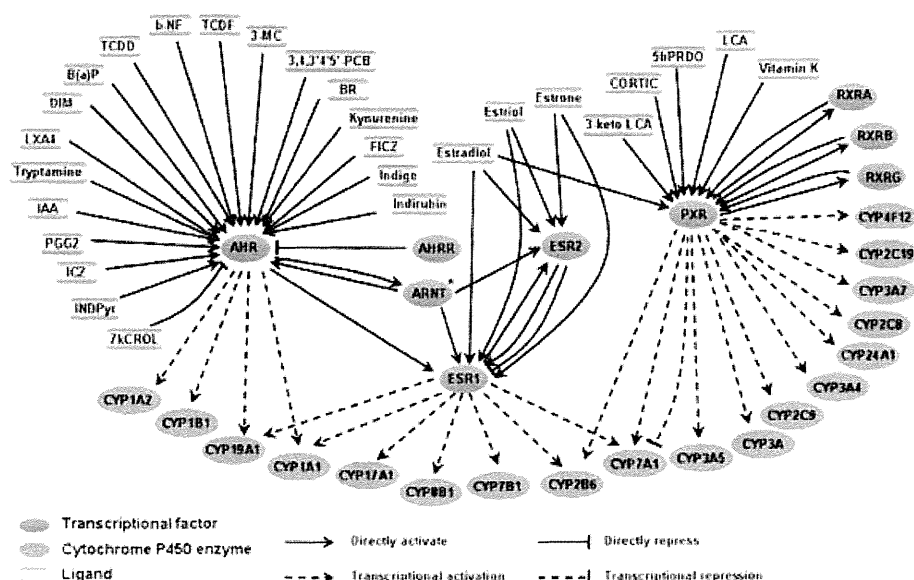
— 136 —

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

Gene	SNP	Genotype	Case (n = 95)		Control (n = 141)		OR (95% CI)	P_{trend}[b]
			No.[a]	%	No.	%		
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

Gene	SNP	Genotype	Case (n = 98)		Control (n = 141)		OR (95% CI)	P_{trend}[b]
			No.[a]	%	No.	%		
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

Gene	SNP	Genotype	Case (n = 58)		Control (n = 129)		OR (95% CI)	P_{trend}[b]
			No.[a]	%	No.	%		
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	SNP7	0.5318	7/10	0.377
		population (SNP4, 6, 7)	SNP6, 7	0.5999	8/10	0.055
			SNP4, 6, 7	0.6281	10/10	0.011
	Combination of JPN and	SNPs significantly related with CO risk in	SNP5	0.5093	9/10	0.828
	ITA population[c]	JPN (SNP4, 6, 7) and ITA population (SNP1, 5)	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	SNP2	0.6958	10/10	0.001
		JPN population (SNP2, 3, 7)	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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