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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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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%

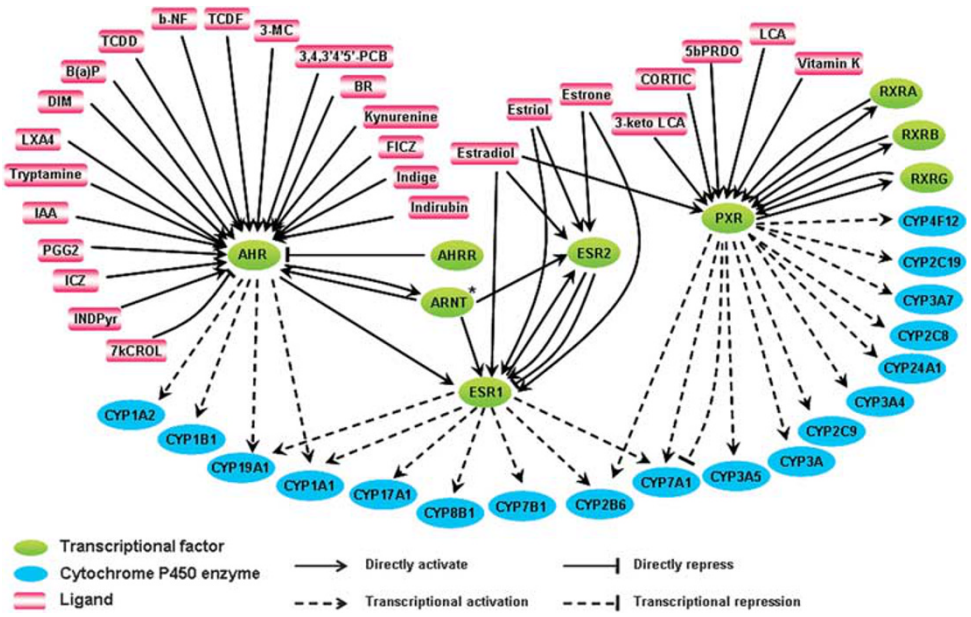

Figure 1 Network-based analysis for molecular interactions of AHR, PXR, ESR1 and ESR2 using KeyMolnet. A gene list of AHR, PXR, ESR1 and ESR2 was imported into KeyMolnet that generated a molecular network composed of 27 ligands (red), 9 transcription factors (green) and 18 cytochrome P450 enzymes (blue). 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 ($P_{\text{trend}}<0.05$).

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 ($P_{\text{trend}}<0.05$). 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 ($P_{\text{trend}} = 0.067$). 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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