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Association of Micropenis with Pro185Ala Polymorphism of the Gene for Aryl Hydrocarbon Receptor Repressor Involved in Dioxin Signaling

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Abstract. The prevalence of undermasculinized external genitalia has increased in several countries including Japan, and this phenomenon has primarily been ascribed to the deleterious effects of environmental endocrine disruptors such as dioxins. To examine a possible role of the genetic susceptibility to dioxins in the development of micropenis (MP), we studied the Arg554Lys polymorphism of the gene for aryl hydrocarbon receptor (*AHR*) and the Pro185Ala polymorphism of the gene for aryl hydrocarbon receptor repressor (*AHRR*), in 73 boys with MP (34 boys with mild MP from –2.1 to –2.5 SD and 39 boys with severe MP below –2.5 SD) and 80 control males (50 boys and 30 fertile adult males). The allele and genotype frequencies of the *AHR* polymorphism were comparable between the two groups of males, but those of the *AHRR* polymorphism were significantly different, with the Pro allele and the Pro/Pro genotype being more frequent in boys with MP than in control males (*P*-value: 0.0029 for the allele frequency and 0.011 for the genotype frequency). In addition, both polymorphisms were comparable in the allele and genotype frequencies between boys with mild MP and those with severe MP and between control boys and control fertile adult males. The results suggest that the *AHRR* Pro185Ala polymorphism may constitute a susceptibility locus for the development of MP in response to dioxins.

Key words: micropenis, dioxin, aryl hydrocarbon receptor, aryl hydrocarbon receptor repressor, polymorphism
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MICROPENIS (MP) is a heterogeneous condition defined as significantly small penis without associated external genital ambiguity such as hypospadias [1, 2]. It is a common ailment and can take place as an isolated form or as a part of impaired male sex development [1, 2]. While MP with other genital features often results from single gene abnormalities, apparently isolated MP, though it still can be caused by single gene abnormalities in rare cases [3, 4], usually occurs as a multifactorial trait subject to various relatively minor genetic and environmental factors [1, 2].

The prevalence of undermasculinized external genitalia has increased during the last few decades at least in several countries including Japan [5, 6]. Similar tendencies have also been observed for spermatogenic failure and testicular cancer [5]. Furthermore, deterioration of male reproductive health is also identified in many wildlife species [7]. It has been hypothesized, therefore, that these adverse changes in males are interrelated events primarily caused by the deleterious effects of environmental endocrine disruptors (EEDs) [5]. Indeed, most EEDs are known to have estrogenic effects that disturb the endocrine balance and affect male reproductive function [5].

Dioxins are environmental contaminants primarily

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produced in the manufacture of chlorinated hydrocarbons. Experimental studies with 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) have indicated that dioxins exert deleterious effects on male reproductive systems, in addition to other diverse disadvantageous biological effects [8]. For example, *in utero* and lactational exposure of male rats to TCDD disturbs development of male reproductive organs and affects spermatogenesis [8]. These findings imply that dioxins belong to EEDs that are disadvantageous to male reproductive health [5].

The detrimental effects of dioxins would depend on individual's susceptibility, in addition to the dosage and the developmental stage of exposure. In this context, the biological effects of dioxins are known to be mediated by several molecules including the aryl hydrocarbon receptor (AHR), the aryl hydrocarbon receptor nuclear translocator (ARNT), and the aryl hydrocarbon receptor repressor (AHRR) [9–12]. Dioxins bind to AHR in the cytoplasm, and the ligand-bound AHR is translocated into the nucleus and dimerizes with ARNT. The ligand-AHR-ARNT complex binds to the cognate xenobiotic responsive elements (XREs) in the promoter/enhancer regions of multiple genes including those involved in the xenobiotic metabolism, and regulates their transcription. AHRR is induced by the AHR-mediated signaling and constitutes a component of a negative feedback loop in the dioxin-related signal transduction pathway; AHRR also dimerizes with ARNT and the AHRR-ARNT complex binds to the XREs in competition with the ligand-AHR-ARNT complex, without altering expression of the target genes [12]. Thus, it is expected that polymorphisms of the genes for these molecules could be relevant to the individual's susceptibility to dioxins.

To date, two informative polymorphisms, Arg554Lys at exon 10 of *AHR* and Pro185Ala at exon 6 of *AHRR*, have been identified in the Japanese population, whereas no useful polymorphism has been detected for *ARNT* in the Japanese population, including the Asp511Asn and Asp517Glu at exon 16 that have been identified in non-Japanese populations [13–15]. In this context, Fujita *et al.* [14] have reported that the frequencies of the Pro allele and the Pro/Pro genotype of *AHRR* Pro185Ala polymorphism are significantly higher in 59 boys with severe MP (<–2.5 SD) than in 80 control males, whereas the prevalence of *AHR* Arg554Lys polymorphism is similar between the two groups of males. To further examine whether the *AHRR* Pro185Ala polymorphism constitutes a susceptibility locus for MP, we studied a different group of boys with MP.

Materials and Methods

Subjects

Seventy-three Japanese boys with MP (age, 0–14 yr; median, 7 yr) were studied. They satisfied the following selection criteria: (1) stretched penile length below –2.0 SD of the mean in age-matched normal Japanese boys [16]; (2) lack of other discernible genital and extragenital features, including hypospadias and gynecomastia; (3) 46,XY karyotype in all the ≥20 lymphocytes analyzed; (4) absence of demonstrable mutation of the genes for 5α-reductase-2 (*SRD5A2*) and androgen receptor (*AR*) that could cause MP [3, 4]; (5) no increase in the allele and genotype frequencies of Val89Leu polymorphism at exon 1 of *SRD5A2* that is known to decrease 5α-reductase-2 activity by ~30% and no expansion of CAG repeat lengths at exon 1 of *AR* that has been shown to be inversely correlated with transactivation function of the *AR* gene [3, 4]; and (6) apparently normal growth and development.

Since –2.0 SD has been regarded as the lower limit of normal variations for most quantitative traits and –2.5 SD has been used as the lower limit of normal penile lengths [1, 2], the 73 boys were divided into two groups: (1) 34 patients with mild MP from –2.1 to –2.5 SD below the mean (age, 0–13 yr; median, 8 yr); and (2) 39 patients with severe MP below –2.5 SD of the mean (age, 0–14 yr; median, 6 yr). Cryptorchidism was present in three boys with mild MP (two bilateral and one unilateral) and in two boys with severe MP (one bilateral and one unilateral), and testis was palpable in the inguinal region in all the five boys with cryptorchidism. Basal serum gonadotropin and testosterone values were within age- and pubertal tempo-matched Japanese reference data in most boys, except for low follicle stimulating hormone levels in a 9-year-old boy with mild MP (0.2 mIU/mL) and in a 7-year-old boy with severe MP (<0.2 mIU/mL).

For controls, the previous data of 50 Japanese boys with apparently normal external genitalia who were diagnosed as having idiopathic short stature (age, 3–16 yr; median, 8.5 yr) and 30 Japanese adult males with proven fertility (age, 25–48 yr; median, 38.0 yr)

were utilized [14]. All the boys with MP and the control males came from the urban or suburb area of Tokyo metropolis and Kawasaki City. They were free from particular residential environments such as the vicinity of chemical factories or farms, specific dietary habits such as vegetarianism or nearly pure meat or fish diet, and intake of drugs with hormonal effects. This study has been approved by the Institutional Review Board Committees at National Center for Child Health and Development. Informed consent was obtained from each subject and/or his parents.

Analysis of Arg554Lys polymorphism in AHR

Leukocyte genomic DNA was analyzed by the 5' nuclease assay with Taqman Minor Groove Binder (MGB) probes on the ABI PRISM 7000 Sequence Detection System (Applied Biosystems, www.appliedbiosystems.com) [17]. The Taqman MGB probe for the arginine allele was labeled with FAM, and that for the lysine allele was labeled with VIC. They were obtained from Applied Biosystems, together with the PCR primers for the amplification of a genomic region encompassing the MGB probe sequence (Assays-On-Demand SNP Genotyping Products; Assay ID: C_11170747_10).

Analysis of Pro185Ala polymorphism in AHRR

Genomic DNA was amplified by PCR with primers flanking exon 6, and the PCR products were digested with *Bbv*I. The methods were as described previously [14]. The PCR products harbor a naturally occurring *Bbv*I site and a polymorphism dependent *Bbv*I site where the proline allele is digested with *Bbv*I and the alanine allele is undigested with *Bbv*I. Thus, the *Bbv*I digestion yields two fragments for the alanine allele and three fragments for the proline allele. The presence of the naturally occurring *Bbv*I site served as an internal control in this method.

Statistical analysis

Statistical significance was examined by Pearson's χ^2 test. $P<0.05$ was considered significant.

Results

Arg554Lys polymorphism in AHR

The data are summarized in Table 1. There was no significant difference in the allele and the three types of genotype frequencies between boys with MP (mild, severe, and total) and control males, as well as between boys with mild MP and those with severe MP and between control boys and control adults (data not shown).

Pro185Ala polymorphism in AHRR

The data are summarized in Table 1. The allele frequencies were significantly different between boys with MP (mild, severe, and total) and control males, with the Pro allele being more prevalent in boys with MP than in control males. Furthermore, at least one of the three types of genotype frequencies was significantly different between boys with MP (mild, severe, and total) and control males, with the highest statistical significance being identified for different types of genotype comparisons. By contrast, no significant difference was identified for the allele and the three types of genotype frequencies between boys with mild MP and those with severe MP and between control boys and control adult males (data not shown).

Discussion

The results provide further support for the previously proposed notion that the Pro allele of *AHRR* Pro185Ala polymorphism raises the susceptibility to the development of severe MP [14], and suggest that the Pro allele is relevant to the occurrence of not only severe MP but also mild MP. Since the highest statistical significance was identified for different types of genotype comparisons between boys with MP (mild, severe, and total) and control males, the Pro allele may exert a co-dominant effect on the development of MP phenotype. By contrast, since no association was identified between MP and the Arg554Lys polymorphism, this polymorphism is unlikely to act as a modifier for the development of MP, as has been suggested previously [14].

The Pro allele of *AHRR* Pro185Ala polymorphism has also been suggested to increase the predisposition to male infertility. Watanabe *et al*. [18] reported that

Table 1. Summary of the association study

		Polymorphism analysis				*P*-value			
		Micropenis			Control (n=80)	M-MP vs. C	S-MP vs. C	T-MP vs. C	M-MP vs. S-MP
		Mild (n=34)	Severe (n=39)	Total (n=73)					
<*AHR*: Arg554Lys>									
Allele freq.	Arg	40	47	87	88	0.59	0.44	0.42	0.86
	Lys	28	31	59	72				
Genotype freq.	Arg/Arg	12	15	27	26	0.84	0.76	0.73	0.95
	Arg/Lys	16	17	33	36				
	Lys/Lys	6	7	13	18				
	Arg/Arg	12	15	27	26	0.77	0.52	0.56	0.78
	Arg/Lys+Lys/Lys	22	24	46	54				
	Arg/Arg+Arg/Lys	28	32	60	62	0.56	0.56	0.47	0.97
	Lys/Lys	6	7	13	18				
<*AHRR*: Pro185Ala>									
Allele freq.	Pro	49	52	101	84	**0.0061**	**0.038**	**0.0029**	0.48
	Ala	19	26	45	76				
Genotype freq.	Pro/Pro	18	16	34	22	**0.023**	0.092	**0.011**	0.53
	Pro/Ala	13	20	33	40				
	Ala/Ala	3	3	6	18				
	Pro/Pro	18	16	34	22	**0.0092**	0.14	**0.014**	0.31
	Pro/Ala+Ala/Ala	16	23	39	58				
	Pro/Pro+Pro/Ala	31	36	67	62	0.085	**0.047**	**0.015**	0.86
	Ala/Ala	3	3	6	18				

AHR: aryl hydrocarbon receptor; AHRR: aryl hydrocarbon receptor repressor; M-MP: mild micropenis; S-MP: severe micropenis; T-MP: total micropenis; and C: control.
Mild micropenis: penile length from –2.1 to –2.5 SD; and severe micropenis: penile length below –2.5 SD.

while the difference in the allele frequency of the *AHRR* polymorphism did not reach a significant level, the Pro/Pro genotype frequency was significantly higher in 123 infertile males than in 112 fertile males. The prevalence of the *AHR* Arg554Lys polymorphism was similar between infertile and fertile males. The results would also imply the relevance of the *AHRR* Pro185Ala polymorphism to the deterioration of male reproductive health.

The Pro allele may exert a weaker negative feedback effect on dioxin signaling than the Ala allele, leading to an enhanced dioxin action. In this regard, it has been shown that an agonist-activated AHR-ARNT heterodimer exerts estrogenic actions via a direct interaction with unliganded estrogen receptors (ERs), whereas the heterodimer exhibits anti-estrogenic activities in the presence of high doses of estrogens and represses estrogen-bound ER function [19]. It may be possible, therefore, that an enhanced dioxin effect results in an exaggerated estrogenic effect in males but not in females. Thus, an enhanced dioxin signaling may result in a disturbed endocrine status such as attenuated gonadotropin secretion in males by exaggerating estrogenic action [20], contributing to the development of MP as well as male infertility. Furthermore, such a sex dimorphism in a hormonal action of dioxins may possibly explain the previous finding that the frequency of *AHRR* Pro185Ala polymorphism as well as *AHR* Arg554Lys polymorphism is similar between 45 females with endometriosis, that is stimulated by estrogens and regarded as a target of EEDs [21, 22], and 108 control females [13].

Several points should be made with respect to the present study. First, the analyzed subjects are still too small in number to allow for a definitive conclusion. Second, there may be some unidentified underlying

genetic and/or environmental difference between the MP boys and the control males. Third, since functional studies have not been performed for the Pro185Ala polymorphism, it remains to be elucidated whether the polymorphism has a direct effect on the dioxin-related signal transductions. Indeed, it is possible that the polymorphism serves as a marker for a true hidden functional polymorphism, or that the positive association between MP and the polymorphism has been obtained just by chance. Lastly, it also remains to be determined whether similar results can be reproduced in other presumably EED-related male reproductive disorders such as cryptorchidism and hypospadias, and in other ethnic groups with an increased prevalence of such disorders. Indeed, although multiple studies have been performed for the two polymorphisms, most studies have focused on the susceptibility to cancers or the induction of *CYP1A1* [23, 24], and only three previous studies have shed a light on hormonal effects of dioxins [13, 14, 18].

Despite the above caveats, the present study suggests that the Pro185Ala polymorphism in *AHRR* may constitute a susceptibility locus for the development of mild and severe forms of MP. This notion awaits further case-control studies in undermasculinized disorders and functional studies of the Pro185Ala polymorphism.

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