this study. When the number of subjects studied is not large and the expected difference is small, actual differences are quite likely to pass undetected. Inconsistent results between early and advanced endometriosis might be attributable to the lack of sufficient numbers and possible misclassification in the early endometriosis group. Alternatively, the observed interactions may have occurred merely by chance.

A second issue is our definition of cases and controls. In accordance with the revised classifications of the American Fertility Society, we defined women without endometriosis as controls and women with early (Stage I–II) and advanced endometriosis (Stage III–IV) as cases,[16] although there is no clear criterion for dichotomizing cases. The present study did not show a persuasive inverse association between urinary isoflavones and the risk of early endometriosis, although a strong protective effect was found for advanced endometriosis. Further analysis, however, did show an inverse association between urinary isoflavones and the severity of endometriosis. This finding may be reasonable given that endometriosis occurs in a continuum of severity.

A third issue is measurement of urinary levels of isoflavones. The present study measured urinary excretion of genistein and daidzein as markers of soy isoflavone consumption. Urinary excretion of soy isoflavones is reportedly related to annual dietary intake of soy isoflavones.[19] Since we collected spot urine samples, intraindividual variation in urinary isoflavones cannot be ignored. Such misclassification, however, is probably nondifferential and would lead to a null result.

Participants in the present study were infertile. They might therefore have changed their diet due to their symptoms or in attempt to become pregnant. If a change in diet was more likely among patients with advanced endometriosis than the controls, our findings might have been the result of the change in diet. In addition, given reports that factors associated with endometriosis differ between parous women (who experienced neither primary nor secondary infertility) and nulliparous infertile women,[30,31] the influence of urinary isoflavone levels on endometriosis risk between the 2 groups may have differed. Therefore, our present findings may be limited to infertile women.

In conclusion, in a case–control study in infertile Japanese women, we found that higher urinary level of isoflavones was associated with a reduced risk of advanced endometriosis. Although the interaction between urinary genistein and *ESR2* gene polymorphisms supported the mechanism for a role of isoflavones in the etiology of endometriosis, further studies with a large number of subjects are needed to confirm these findings.

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Interaction between cytochrome P450 gene polymorphisms and serum organochlorine TEQ levels in the risk of endometriosis

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Exposure to dioxins and polychlorinated biphenyls (PCBs) has been suggested as a possible etiologic factor for endometriosis, but the association remains highly controversial. To assess whether cytochrome P450 (CYP) gene polymorphisms modulate the effect of dioxins and/or PCBs in endometriosis risk, we conducted a case–control study among infertile Japanese women. A total of 138 eligible women aged 20–45 were diagnosed laparoscopically and classified into three subgroups: control (no endometriosis), early endometriosis (stages I–II) and advanced endometriosis (stages III–IV). Neither CYP1A1 Ile462Val and CYP1B1 Leu432Val polymorphisms (genotypes with versus genotypes without the minor allele) nor serum dioxin and PCB toxic equivalency (TEQ) levels (low versus high) were independently associated with either early or advanced endometriosis risk. However, genotypes with the CYP1A1 462Val allele showed a statistically significant reduced risk of advanced endometriosis in combination with high serum dioxin TEQ levels (adjusted odds ratio = 0.13, 95% confidence interval: 0.02–0.76) (*P* for interaction = 0.08). Although no association was found between serum PCB TEQ level and advanced endometriosis in any stratum of CYP1B1 Leu432Val polymorphism, a statistically significant interaction was found (*P* for interaction = 0.05). CYP1A1 and CYP1B1 polymorphisms may modify the relation between environmental exposure to organochlorine and advanced endometriosis risk.

Keywords: CYP1A1/CYP1B1/endometriosis/gene–environment interaction/organochlorine

Introduction

Exposure to certain xenoestrogens, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) and polychlorinated biphenyls (PCBs) has been suggested as a possible etiologic factor for endometriosis. The association between endometriosis and these organochlorines has been the subject of a number of studies (Mayani *et al.*, 1997; Lebel *et al.*, 1998; Pauwels *et al.*, 2001; Eskenazi *et al.*, 2002; Heilier *et al.*, 2005; Louis *et al.*, 2005; Tsukino *et al.*, 2005), but remains highly controversial. At this time, there is insufficient evidence to establish a definitive link between endometriosis and organochlorine exposure.

Endometriosis, an estrogen-dependent disease, is regarded as a complex trait influenced by both genetic and environmental factors (Kennedy, 1998). To understand this condition, consideration must be given to both the individual contributions of genetic and environmental factors and their magnitude, and also the interactions of these factors. Gene–environment interactions, the multiplicative joint effects of genetic predisposition and environmental factors, are important in understanding how risk factors act together and in identifying high-risk groups (Brennan, 2002).

Genetic polymorphisms in cytochrome P450 (CYP) 1A1 and CYP1B1 are putative genetic factors associated with inter-individual susceptibility to organochlorines. CYP1A1 and CYP1B1 are phase I drug-metabolizing enzymes that are critical to both xenobiotic and estrogen metabolism. The activities of CYP1A1 and CYP1B1 are determined jointly by genetic and environmental factors (Gonzalez, 1988; Martucci and Fishman, 1993). Inconsistent associations between endometriosis and organochlorine exposure might be attributable to the different genetic susceptibilities in the populations studied.

The magnitude of risk associated with gene–environment interactions can be estimated from a case–control study. In the present study, we tested the hypothesis that the genetic polymorphisms CYP1A1 Ile462Val and CYP1B1 Leu432Val modulate the effect of dioxins and/or PCBs in the risk of endometriosis, and thereby assessed the possibility that altered risk arises from genetic predisposition.

Materials and Methods

Study population

This study was part of a case–control study conducted on a Japanese population to investigate associations between genetic and environmental factors

in endometriosis (Tsukino *et al.*, 2005). Consecutive female patients aged 20–45 who attended the Department of Obstetrics and Gynecology at Jikei University School of Medicine Hospital for infertility between 1999 and 2000 were recruited. Since pregnancy commonly results in complete resolution of minimal or mild endometriosis, women who had given birth or lactated were ineligible, leaving a total of 159 women who met the criteria. After excluding 15 women who did not give consent, 5 who did not undergo blood screening or laparoscopic examination and 1 whose DNA sample was not available, a total of 138 women were available for the study (participation rate = 87%). No participants had undergone prior empiric therapy before laparoscopic examination.

All study protocols were approved by the Institutional Review Boards of Jikei University, National Cancer Center, University of Miyazaki, National Institute for Environmental Studies and the US Centers for Disease Control and Prevention (CDC). All participants provided written informed consent before laparoscopic examination.

Before the laparoscopic examination, participants were interviewed by a single trained interviewer using a structured questionnaire. Participants also gave a fasting blood sample before the laparoscopic examination. Blood samples were divided into plasma and buffy layers and stored at −80 C until analysis.

Diagnosis of endometriosis

In the present study, all participants underwent diagnostic laparoscopy as part of an infertility work-up. Laparoscopy is essential to the accurate diagnosis of endometriosis because one-third of women with endometriosis are asymptomatic (Rawson, 1991). The degree of endometriosis was diagnosed according to the Revised American Fertility Society (r-AFS) classification (American Fertility Society, 1985) and/or histologic diagnosis. Endometriosis was absent in 59 women (43%), stage I in 21 women (15%), stage II in 10 women (7%), stage III in 23 women (17%) and stage IV in 25 women (18%). Current theories of endometriosis suggest that what is defined as minimal/mild endometriosis may actually represent a normal physiologic process. Furthermore, a lack of consistency between laparoscopic and histologic diagnosis has been reported, particularly for minimal/mild endometriosis (Marchino *et al.*, 2005). Considering the more severe stages as a separate category thus appears reasonable (Zondervan *et al.*, 2002). Although women without endometriosis and with stage I were designated as controls and women with stage II or more severe endometriosis were designated as cases in the previous study (Tsukino *et al.*, 2005), considering the current theories of endometriosis mentioned earlier, we classified cases into two subgroups in the present study: early (stages I–II) or advanced endometriosis (stages III–IV). Women without endometriosis were defined as controls. Among controls, several conditions known to cause infertility were confirmed laparoscopically, including myoma of the uterus (34%) and polycystic ovary (19%).

DNA extraction and genotyping

Genomic DNA was extracted from peripheral white blood cells using a conventional protease K method. CYP1A1 Ile462Val (dbSNP rs1048943) and CYP1B1 Leu432Val (dbSNP rs1056836) polymorphisms were genotyped using PCR-RFLP analysis as described previously (Huang *et al.*, 1999; Tang *et al.*, 2000). Genotyping was conducted by laboratory personnel blinded to case–control status. To validate the genotyping, duplicate samples from some patients were provided in a manner blinded to the laboratory personnel; concordance for the blinded samples was 100%. Thus, experimenter bias was demonstrably minimized.

Measurement of organochlorines

Serum organochlorines were measured as described in our previous study (Tsukino *et al.*, 2005). Briefly, analyses were performed at the US CDC using gas chromatography/high-resolution isotope dilution mass spectrometry for 58 compounds: 8 polychlorinated dibenzo-*p*-dioxin (PCDDs), 10 polychlorinated dibenzo-*p*-furans (PCDFs), 4 coplanar PCBs (cPCBs) and 36 ortho-substituted PCBs. The serum levels for these compounds were adjusted for serum lipid levels.

The term dioxins refers collectively to a group of PCDDs, PCDFs and cPCBs. To calculate the toxic equivalency (TEQ) of these compounds, a TEQ factor (TEF) was assigned to each of the PCDDs, PCDFs and cPCBs (Van den Berg *et al.*, 1998). Summation of the TEQs of PCDDs, PCDFs and cPCBs gives the TEQ of dioxins (pg TEQ/g lipid). In contrast, most of the PCBs are assigned a TEF of zero. Summation of the TEQs of mono-*ortho*-substituted PCBs (mPCBs) gives the TEQ of PCBs (pg TEQ/g lipid).

Statistical analysis

CYP1A1 Ile462Val and CYP1B1 Leu432Val polymorphisms were classified into two subgroups: genotypes homozygous for the major allele (CYP1A1: Ile/Ile; CYP1B1: Leu/Leu) and pooled heterozygous and minor allele homozygous genotypes (CYP1A1: Ile/Val and Val/Val; CYP1B1: Leu/Val and Val/Val). Concentrations of lipid-adjusted serum dioxins and PCB TEQ levels were defined as *low* or *high* based on the median value of control subjects.

To assess the main genetic and environmental effects on endometriosis, odds ratios (ORs) and their 95% confidence intervals (95% CIs) were calculated independently for CYP gene polymorphisms and serum levels of organochlorines using multivariate logistic regression analyses. To control for possible confounding factors, age was adjusted for in the multivariable logistic regression models. Secondly, risks of endometriosis were compared by a stratified model of genetic polymorphisms and organochlorine exposure. Multiplicative interactions were assessed by introducing a cross-product term between two-category genotypes and levels of serum organochlorines into the logistic regression models.

A two-sided $P < 0.05$ was considered significant in the analysis of main effects, whereas $P < 0.1$ was used when testing for the presence of interactions. SPSS for Windows software (version 11.0, SPSS JAPAN, Tokyo, Japan) was used for statistical analysis.

Results

Table 1 shows baseline characteristics of cases and controls. No significant difference was seen in mean age or body mass index between groups, or in the distribution of menstrual bleeding, hypermenorrhea and smoking. The advanced endometriosis group displayed a significantly shorter menstrual cycle than controls (controls, 30.7 ± 6.1 days; advanced endometriosis, 28.3 ± 3.0 days; $P = 0.01$) and was more likely to have menstrual cramp and dyspareunia.

The distributions of CYP gene polymorphisms and serum organochlorine levels are shown in Table 2. The genotypic distributions of CYP1A1 Ile462Val and CYP1B1 Leu432Val polymorphisms were concordant with Hardy–Weinberg equilibrium (χ^2 test: CYP1A1 Ile462Val, $P = 0.42$; CYP1B1 Leu432Val, $P = 0.52$). In the statistical analyses of dioxins and PCBs, we excluded one sample from each because the serum concentrations could not be reliably measured due to sample shortage. The range of concentrations among all subjects was 3.39–38.33 pg TEQ/g lipids for serum dioxins and 0.00–7.55 pg TEQ/g lipids for PCBs. Median values of serum dioxin and PCB concentrations were 18.18 and 1.21 pg TEQ/g lipids for controls, 17.69 and 1.05 pg TEQ/g lipids for early endometriosis and 16.03 and 1.11 pg TEQ/g lipids for advanced endometriosis, respectively.

In the present study, there were no independent associations between the CYP gene polymorphisms and risk of either early or advanced endometriosis (Table 2). Although serum dioxin levels showed a non-significant inverse association with advanced endometriosis (Table 2, adjusted OR: 0.46, 95% CI: 0.20–1.06), no other associations were seen between serum organochlorines and either early or advanced endometriosis. Further adjustment for menstrual cycle and duration of menstrual bleeding did not substantially affect the results (data not shown).

To assess possible effect modifications by CYP gene polymorphisms, we evaluated the association between serum organochlorine TEQ levels and risk of endometriosis stratified by CYP genotypes. No interaction between serum organochlorine level and CYP genotype was observed in early endometriosis (Tables 3 and 4). On the other hand, the CYP1A1 Ile462Val pooled Ile/Val and Val/Val genotypes

Table 1: Baseline characteristics of cases and controls

Number of subjects	Controls ($n = 59$)	Endometriosis			
		Early (stages I–II) ($n = 31$)	*P* for difference	Advanced (stages III–IV) ($n = 48$)	*P* for difference
Age (years), mean ± SD	33.1 ± 4.1	32.3 ± 3.2	0.35	32.6 ± 3.7	0.52
Body mass index (kg/m^2), mean ± SD	21.0 ± 3.4	20.6 ± 2.1	0.47	20.2 ± 2.1	0.12
Menstrual cycle (days), mean ± SD	30.7 ± 6.1	29.6 ± 3.6	0.34	28.3 ± 3.0	0.01
Menstrual bleeding, no. (%)			0.79		0.65
<7 days	42 (71)	21 (68)		35 (73)	
≥7 days	15 (25)	6 (19)		10 (21)	
Missing	2 (3)	4 (13)		3 (6)	
Hypermenorrhea, no. (%)			0.80		0.83
No	39 (66)	20 (65)		29 (60)	
Yes	18 (31)	7 (23)		15 (31)	
Missing	2 (3)	4 (13)		4 (8)	
Menstrual cramping, no. (%)			0.32		0.02
No	10 (17)	2 (6)		1 (2)	
Yes	47 (80)	25 (81)		44 (92)	
Missing	2 (3)	4 (13)		3 (6)	
Dyspareunia, no. (%)			0.48		<0.01
No	31 (53)	12 (39)		10 (21)	
Yes	25 (42)	14 (45)		34 (71)	
Missing	3 (5)	5 (16)		4 (8)	
Smoking status, no. (%)			0.81		>0.99
Never smoker	38 (64)	19 (61)		29 (60)	
Current or ever smoker	19 (32)	8 (26)		15 (31)	
Missing	2 (3)	4 (13)		4 (8)	

showed a statistically significant reduced risk of advanced endometriosis in combination with a high serum dioxin TEQ level (Table 3, adjusted OR: 0.13, 95% CI: 0.02–0.76). There was a statistically significant interaction between the CYP1A1 Ile462Val polymorphism and serum dioxin TEQ level (Table 3, *P* for interaction = 0.08). Although no association was found between serum PCB TEQ level and advanced endometriosis in any stratum of CYP1B1 Leu432Val polymorphism, a statistically significant interaction was noted (Table 4, *P* for interaction = 0.05). For advanced endometriosis, no interaction was found in other combinations of CYP gene polymorphism and serum organochlorine level (Tables 3 and 4).

Discussion

In the present study, we demonstrated statistically significant interactions between the CYP1A1 Ile462Val and CYP1B1 Leu432Val polymorphisms and serum organochlorine TEQ levels in the risk of advanced endometriosis. This interaction would suggest the presence

Table 2: Effects of CYP gene polymorphisms and serum organochlorine levels considered separately in the risk of endometriosis

	Controls (n)	Endometriosis			
		Early (stages I–II)		Advanced (stages III–IV)	
		n	Adjusted ORs (95% CI)[a]	n	Adjusted ORs (95% CI)[a]
CYP gene polymorphism					
CYP1A1 Ile462Val					
Ile/Ile	40	19	1	31	1
Ile/Val, Val/Val	19	12	1.35 (0.54–3.37)	17	1.20 (0.53–2.71)
CYP1B1 Leu432Val					
Leu/Leu	40	21	1	34	1
Leu/Val, Val/Val	19	10	1.01 (0.40–2.71)	14	0.90 (0.39–2.06)
Serum organochlorines[b]					
Dioxins					
Low (<18.18)	29	17	1	32	1
High (≥18.18)	30	14	0.93 (0.36–2.41)	15	0.46 (0.20–1.06)
PCBs					
Low (<1.21)	29	17	1	27	1
High (≥1.21)	30	13	0.84 (0.33–2.14)	21	0.80 (0.35–1.81)
Total dioxins/ PCBs					
Low (<20.32)	29	17	1	32	1
High (≥20.32)	30	13	0.84 (0.33–2.16)	15	0.47 (0.21–1.05)

[a]Adjusted for age.
[b]pg TEQ/g lipids.

Table 3: Effect modifications of the association between endometriosis and serum organochlorine levels by CYP 1A1 gene polymorphism

CYP gene polymorphism	Serum organochlorines[a]	Controls (*n*)	Endometriosis			
			Early (stages I–II)		Advanced (stages III–IV)	
			n	Adjusted ORs (95% CI)[b]	*n*	Adjusted ORs (95% CI)[b]
CYP1A1 Ile462Val	Dioxins					
Ile/Ile	Low (<18.18)	20	9	1	18	1
Ile/Ile	High (≥18.18)	20	10	1.10 (0.32–3.75)	13	0.75 (0.28–2.06)
Ile/Val, Val/Val	Low (<18.18)	9	8	1	14	1
Ile/Val, Val/Val	High (≥18.18)	10	4	0.63 (0.13–3.15)	2	0.13 (0.02–0.76)*
P for interaction				0.30		0.08†
CYP1A1 Ile462Val	PCBs					
Ile/Ile	Low (<1.21)	19	10	1	19	1
Ile/Ile	High (≥1.21)	21	9	0.73 (0.21–2.56)	12	0.57 (0.20–1.62)
Ile/Val, Val/Val	Low (<1.21)	10	7	1	8	1
Ile/Val, Val/Val	High (≥1.21)	9	4	0.65 (0.13–3.28)	9	1.38 (0.36–5.34)
P for interaction				0.68		0.36
CYP1A1 Ile462Val	Total dioxins/PCBs					
Ile/Ile	Low (<20.32)	19	10	1	19	1
Ile/Ile	High (≥20.32)	21	9	0.75 (0.23–2.51)	12	0.58 (0.21–1.58)
Ile/Val, Val/Val	Low (<20.32)	10	7	1	13	1
Ile/Val, Val/Val	High (≥20.32)	9	4	0.89 (0.17–4.55)	3	0.27 (0.06–1.27)
P for interaction				0.74		0.38

[a]pg TEQ/g lipids.
[b]Adjusted for age.
*$P < 0.05$ for main effects.
†$P < 0.1$ for interaction terms.

of an underlying biologic effect modification, possibly resulting in an altered disease phenotype. The results of this study provide epidemiologic clues to the etiology and pathogenesis of endometriosis and identify populations at altered risk because of CYP gene polymorphisms and serum organochlorine TEQ levels.

Genetic factors were implicated in endometriosis by a large study in twins, which found that 51% of the variance of susceptibility may be attributable to genetic influences (Treloar *et al.*, 1999). The effect of single genetic or environmental factors is usually weak; rather, multiple genetic and environment factors collaboratively contribute to the phenotypic variation of endometriosis. Indeed, the analysis of gene–environment interactions in our present study identified synergistic effects between CYP gene polymorphisms and serum organochlorines, although genetic or environmental factors alone did not cause statistically significant differences in the risk of endometriosis.

Table 4: Effect modifications of the association between endometriosis and serum organochlorine levels by CYP1B1 gene polymorphism

CYP gene polymorphism	Serum organochlorines[a]	Controls (*n*)	Endometriosis			
			Early (stages I–II)		Advanced (stages III–IV)	
			n	Adjusted ORs (95% CI)[b]	*n*	Adjusted ORs (95% CI)[b]
CYP1B1 Leu432Val	Dioxins					
Leu/Leu	Low (<18.18)	20	12	1	23	1
Leu/Leu	High (≥18.18)	20	9	0.84 (0.27–2.62)	11	0.53 (0.20–1.41)
Leu/Val, Val/Val	Low (<18.18)	9	5	1	9	1
Leu/Val, Val/Val	High (≥18.18)	10	5	1.20 (0.21–6.87)	4	0.35 (0.07–1.63)
P for interaction				0.81		0.84
CYP1B1 Leu432Val	PCBs					
Leu/Leu	Low (<1.21)	18	11	1	22	1
Leu/Leu	High (≥1.21)	22	9	0.73 (0.23–2.28)	12	0.49 (0.18–1.38)
Leu/Val, Val/Val	Low (<1.21)	11	6	1	5	1
Leu/Val, Val/Val	High (≥1.21)	8	4	1.15 (0.21–6.25)	9	2.36 (0.56–10.0)
P for interaction				0.72		0.05†
CYP1B1 Leu432Val	Total dioxins/PCBs					
Leu/Leu	Low (<20.32)	20	12	1	23	1
Leu/Leu	High (≥20.32)	20	8	0.72 (0.23–2.25)	11	0.52 (0.20–1.39)
Leu/Val, Val/Val	Low (<20.32)	9	5	1	9	1
Leu/Val, Val/Val	High (≥20.32)	10	5	1.20 (0.21–6.87)	4	0.35 (0.07–1.63)
P for interaction				0.70		0.84

[a]pg TEQ/g lipids.
[b]Adjusted for age.
†$P < 0.1$ for interaction terms.

In this study, we found that the presence of a CYP1A1 462Val allele was associated with a statistically significant decreased risk of advanced endometriosis among women with high serum dioxins. The CYP1A1 462Val allele has been reported to be positively associated with TCDD-induced CYP1A1 mRNA expression (Landi *et al.*, 2005). The most plausible hypothesis to explain our results is that sustained activation of CYP1A1 by dioxins alters estrogen metabolism, resulting in a lower susceptibility to endometriosis.

Significant interactions indicate that the effect of organochlorines differ between two strata of CYP gene polymorphism. In contrast to the relationship between CYP1A1 Ile462Val and serum dioxin TEQ, we observed a statistically significant interaction between CYP1B1 Leu432Val polymorphism and serum PCB TEQ, and the CYP1B1 432Val allele seemed to be associated with an increased risk of endometriosis in combination with a high serum PCB TEQ. Considering the relatively small number of subjects, the statistically significant interactions observed may have occurred merely by chance. Intrinsically, some PCBs have estrogenic properties whereas dioxins and dioxin-like PCBs have antagonistic effects (Toppari *et al.*, 1996). The effect of organochlorines in individuals might be defined by the variety of different activations by CYP gene polymorphisms.

The frequency of CYP1A1 Ile462Val and CYP1B1 Leu432Val polymorphisms is known to vary widely in different populations (Solus *et al.*, 2004). The discrepancy in previous studies of organochlorine exposure and endometriosis may arise in part from inter-individual variability in susceptibility to organochlorines and to a dose-related bimodal effect (Yang *et al.*, 2000). The CYP1A1 Ile462Val and CYP1B1 Leu432Val polymorphisms may be a useful genetic marker predicting susceptibility to dioxins. It is preferable to include both genetic and environmental assessment in the study of complex traits.

The present study showed statistically significant interactions between CYP gene polymorphisms and serum organochlorine TEQ levels in the risk of advanced endometriosis, but not in that of early endometriosis. This apparent inconsistency might be attributable to diagnostic bias in early endometriosis, as mentioned in Methods earlier. If early endometriosis reflects normal physiology rather than 'real endometriosis', it would lead to a null result. In this regard, we clarified the effect of case and control definitions on the results by repeating the analyses in Tables 2–4 using the previous definition by Tsukino *et al.* (2005), namely control (no endometriosis and stage I) and cases (stages II–IV). However, these additional analyses did not change the results, and the definition of cases and controls had no effect on our conclusions.

Although this is probably the first study of CYP gene polymorphisms and organochlorines in endometriosis, the CYP1A1 462Val allele has been reported to be mainly associated with increased risk of post-menopausal breast cancer in women with high serum PCBs (Moysich *et al.*, 1999; Laden *et al.*, 2002; Zhang *et al.*, 2004; Li *et al.*, 2005). This discrepancy between breast cancer and endometriosis may be attributable to different effects of organochlorines on carcinogenesis (Whysner and Williams, 1996), different responsiveness of mammary gland and endometrium (Gottardis *et al.*, 1988) and the interaction of serum organochlorines with estradiol levels (Ohtake *et al.*, 2003). Further, more detailed molecular studies are needed to clarify the relationships between CYP gene polymorphism and organochlorines in the risk of endometriosis.

Participants in the present study were infertile. Given previous reports that factors associated with endometriosis differed between parous women, who experienced neither primary nor secondary infertility, and nulliparous infertile women (Missmer *et al.*, 2004a,b), our present findings may be limited to infertile women. In addition, the use of infertile women as the control group should also be considered. When the study population comprises infertile women only, comparing infertile cases with a control group comprising infertile women without endometriosis may yield results very different from those that would be observed when comparisons are made with fertile women without endometriosis (Signorello *et al.*, 1997). This is particularly true when the exposure of interest, such as menstrual cycle characteristics or reproductive history, is correlated with endometriosis and infertility. As a result of this use of infertile women as the control group, an association between serum organocholorines and the risk of endometriosis might be weakened or masked. Further, we cannot rule out the possibility that serum organocholorines are associated with both endometriosis and infertility.

The major limitation of this study was the small sample size, which limits its statistical power. A larger sample size would allow a more precise estimate of main effects and interactions. Therefore, our results should be interpreted with caution. After reanalysis using a case-only design, which is an efficient and valid method for screening gene–environment interactions (Yang *et al.*, 1997), however, the interaction term between CYP 1A1 Ile462Val polymorphism and serum dioxin level was calculated as 0.045. Measurement of serum estradiol and its CYP1A1/1B1 metabolites would allow further refinement of the association between CYP1A1 and CYP1B1 gene polymorphisms and serum organochlorines, as well as any drug–drug interaction between serum estradiol and organochlorines, in the risk of endometriosis.

In conclusion, this study suggests that the CYP1A1 Ile462Val polymorphism is an effect modifier of the relationship between serum dioxins and the risk of advanced endometriosis. The CYP1B1 Leu432Val polymorphism modulates the effect of PCBs in the risk of advanced endometriosis. Better understanding of the relationships between genetic and environmental factors in complex traits may enable the prediction of widely differing risks of individuals or populations. Genetic susceptibility to the effects of organochlorines may affect a woman's likelihood of developing endometriosis.

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Original Article

Urinary Bisphenol-A Concentration in Infertile Japanese Women and Its Association with Endometriosis: A Cross-Sectional Study

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Abstract

Objectives: Bisphenol A (BPA), a raw material commonly used in the manufacture of resins such as polycarbonate and epoxy, is a possible xenoestrogen that is hypothesized to disrupt the human endocrine system. Humans are widely exposed to BPA. We investigated the urinary concentration of BPA in infertile Japanese women and its possible association with endometriosis.

Materials and Methods: We recruited 166 women (aged 20–45) who had complained of infertility and visited a university hospital in Tokyo. The subjects were interviewed and their urine samples were obtained prior to a laparoscopic diagnosis of endometriosis between January 2000 and December 2001. Urinary total BPA concentration in 140 eligible urine samples was then measured using enzymatic deconjugation of glucuronide and sulfate and high-performance liquid chromatography isotope-dilution tandem mass spectrometry.

Results: Median (25th–75th percentile) unadjusted and creatinine-adjusted urinary BPA concentrations were 1.6 (0.69–2.8) μg/L and 0.80 (0.45–1.3) μg/g creatinine. No significant monotonic association of endometriosis with urinary BPA concentration was observed. Median urinary BPA concentration in women with stage 0–I endometriosis (0.74 μg/g creatinine) did not significantly differ from that in those with stage II–IV endometriosis (0.93 μg/g creatinine) (p for difference=0.24).

Conclusions: This study, based on a larger number of samples than those in previous studies in Japan and using the most reliable analytical method currently available, showed that urinary concentrations of BPA in women who consulted a physician for infertility were not higher than those in other populations. Moreover, no association between urinary BPA concentration and endometriosis was found in this cross-sectional study.

Key words: endocrine disruptor, HPLC-MS/MS, epidemiology, urine, xenoestrogen

Introduction

Bisphenol A (4,4'-isopropylidenediphenol, BPA) is a raw material commonly used in the manufacture of resins such as polycarbonate and epoxy. Polycarbonate plastics are used in certain tableware products and bottles, whereas epoxy resin is used as a protective coating on food and beverage cans (1, 2). Because of its widespread use, humans can be exposed to BPA on a daily basis. BPA has shown estrogenic activity in experimental studies, and is considered a possible xenoestrogen that is hypothesized to disrupt the human endocrine system (2). This in turn has led to scientific and public concern about its effects on human health (3–5), particularly on the issue of whether human exposure to BPA is associated with estrogen-dependent diseases such as endometriosis.

Urinary BPA level has been surveyed in various populations. BPA is frequently detected in urine, which, unlike blood, allows for estimates of daily intake. Most studies to date have been constrained by two problems, however. First, despite the finding that BPA sulfate is a major metabolite of BPA in women (6), to our knowledge only the most recent US study (n=30) employed BPA detection using both mass spectrometry and deconjugation of BPA sulfate (7). Second, sample sizes have been limited to smaller than 100. Thus, taking those conducted

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in Japan as examples, in one study, BPA concentrations was measured in single spot urine collected from 48 female students using electrochemical detection (8); in another study, BPA concentration was measured in 56 pregnant women by enzyme-linked immunosorbent assay (9); and in another study, daily urinary BPA excretion was measured in 36 male university students after deconjugation of BPA glucuronide (10).

Here, we conducted a hospital-based, cross-sectional study to investigate urinary concentrations of BPA in a large sample of infertile Japanese women. The urinary total concentration of BPA was measured using high-performance liquid chromatography isotope-dilution tandem mass spectrometry (isotope-dilution LC-MS/MS). The data obtained were then used to investigate a possible association between urinary BPA concentration and endometriosis stage.

Materials and Methods

Subjects

Subjects were recruited from among 166 consecutive women aged 20 to 45 years who had complained of infertility and consulted the Department of Obstetrics and Gynecology of the Jikei University School of Medicine for treatment of infertility. A total of 148 agreed to participate and provided written informed consent. Women who had previously given birth or who had lactated, and those who had undergone surgery for endometriosis were excluded. One woman of non-Japanese race and another who had lived abroad were also excluded, finally leaving 142 women eligible to participate in this study. Of these, 140 who submitted an eligible single spot urine sample and who underwent laparoscopic examination between January 2000 and December 2001 were available for analysis. Nine patients did not actually complain of infertility according to their questionnaire responses but were included to increase statistical power. The present study was approved by the Institutional Review Boards of the Jikei University School of Medicine and National Cancer Center.

The severity of endometriosis was diagnosed using laparoscopy and then classified into five stages on the basis of the revised American Fertility Society classification (11): stage 0 (n=60), I (n=21), II (n=10), III (n=24), and IV (n=25).

In addition, participants were interviewed before laparoscopic examination by a single trained interviewer using a structured questionnaire to collect information on demographic factors, age, height, weight, personal and family medical, reproductive and menstrual histories, oral contraceptive use, food- and alcohol-consumption frequencies, and smoking history. The items in the questionnaire and participant profile have been described in detail elsewhere (12, 13). No statistically significant differences between stage 0–I and II–IV patients were observed among these profile items except with regard to the regularity of menstrual cycle and average menstrual cycle length.

The participants also collected a first-morning urine sample into a paper cup, which was then transferred into a plastic tube, before laparoscopic examination. Four urine samples were not first-morning urine but were included. Samples were stored at −80°C for about 5 years until analysis but were thawed and refrozen several times during this period.

Urinary BPA analysis

We analyzed urine samples without information on the participant's endometriosis status. Urinary BPA was deconjugated using a hydrolytic enzyme, β-glucuronidase/sulfatase (from *Helix pomatia*), separated using solid-phase extraction and high-performance liquid chromatography, and detected and measured using isotope-dilution tandem mass spectrometry. The analytical method is described in detail in the Appendix. The lower limits of detection (LODs) were 0.30–0.55 μg/L. Accuracy was assured by analyzing blank and quality control samples (2.4 μg/L) along with unknown samples in each analytical batch. Intraday and interday reproducibilities (CV= 8.8% and 19%, n=5, respectively) were previously checked by repeated analyses. The mean total surrogate recovery was 72% in one batch (n=5). In addition, an aliquot of each urine sample was shipped to a commercial clinical examination center, SRL (Tokyo, Japan), for measurement of creatinine concentration. Creatinine was detected in all samples. Urinary BPA concentration [μg/L] was divided by individual creatinine concentration to correct variability in urine dilution, and finally converted into daily BPA intake using equations 1–3 (14).

$$Intake_{BPA} = \frac{(C_{BPA} / C_{creatinine}) \times CE}{f} \times \frac{MW_{dosed}}{MW_{excreted}} \quad (1)$$

$$CE = PRCr / \text{body weight} / 1000 \quad (2)$$

$$PRCr = -4.72 \times \text{age} + 8.58 \times \text{body weight} + 5.09 \times \text{height} - 74.95 \quad (3)$$

where $Intake_{BPA}$ [μg/kg body weight/day] is BPA daily intake; C_{BPA} [μg/L] and $C_{creatinine}$ [g/L] are the individual urinary raw concentrations of BPA and creatinine, respectively; CE [g/kg body weight/day] is the individual urinary daily creatinine-excretion rate to body weight [kg]; MW_{dosed} and $MW_{excreted}$ are the molecular weights of ingested and excreted BPA, respectively; and f is the molar fraction of urinary excreted BPA relative to ingested BPA. CE was calculated using equation 2 on the basis of the individual daily urinary creatinine excretion rate ($PRCr$ [mg/day]), which was predicted using participants' individual age [years], body weight [kg], and height [cm], along with equation 3, an existing multiple regression model (15). Because urinary conjugated BPAs were enzymatically deconjugated to the free form, $MW_{dosed}/MW_{excreted}$ is equal to 1. For BPA, $f = 1$ was also assumed on the basis of the results of two studies of BPA oral administration in humans (16, 17). Daily urinary BPA excretion is considered as average daily BPA intake (3, 10). These unadjusted and creatinine-adjusted concentrations and estimated daily intake were used in statistical analyses.

Statistical analysis

For measurement concentrations of BPA below the LOD, we assigned a value equal to the LOD divided by the square root of 2 (18). For other missing information, we performed list-wise deletion in every analysis. All statistical tests were

two-sided. Statistical analyses were performed using statistical analysis software, SAS version 9.1 (SAS Institute, Cary, NC).

Results

The participants were urban residents aged 24–43 years (median, 32). The median body weight was 51 kg. Among other variables, 63% were white collar workers, 15.7% were current smokers, 10.0% were daily alcohol drinkers, 90.1% had a history of menstrual pain, 57.8% had a history of dyspareunia, 30.8% had a history of hypermenorrhea, and 38.2% had a history of myoma of the uterus. No women had a history of cervical cancer, galactorrhea, adrenal disorder, or diabetes. Endometriosis stage was not associated with any factor except menstrual cycle length, regularity of menstrual cycle, and history of dyspareunia.

BPA was detected in 93% of urine samples. Urinary concentrations of unadjusted and creatinine-adjusted BPA and estimated daily BPA intake are summarized in Table 1, with median values (25th–75th percentiles) of 1.6 (0.69–2.8) μg/L, 0.80 (0.45–1.3) μg/g creatinine, and 0.016 (0.010–0.026) μg/kg body weight/day, respectively. Urinary BPA concentration showed a skewed distribution (Fig. 1), and no association was observed for any of the lifestyle factors described above, such as occupation or smoking status.

Table 1 also shows a cross-sectional comparison of urinary BPA concentration with the stage of endometriosis. Higher unadjusted concentrations were associated with a more advanced stage of endometriosis, although this association was without statistical significance (p for difference=0.08) and became null after division of unadjusted concentrations by urinary creatinine concentration. Overall, we observed no association between endometriosis and any measured concentration of BPA.

Discussion

We measured the urinary concentration of BPA in women consulting a physician for infertility, and found no association between urinary BPA concentration and endometriosis. To our knowledge, this cross-sectional study has the largest sample size for the investigating urinary concentrations of BPA in Japan. It is further notable for its measurement of urinary total concentration of BPA by enzymatic deconjugation of both its glucuronide and sulfate and isotope-dilution LC-MS/MS. In contrast, most previous studies were carried out by either mass spectrometry or deconjugation of BPA sulfate alone.

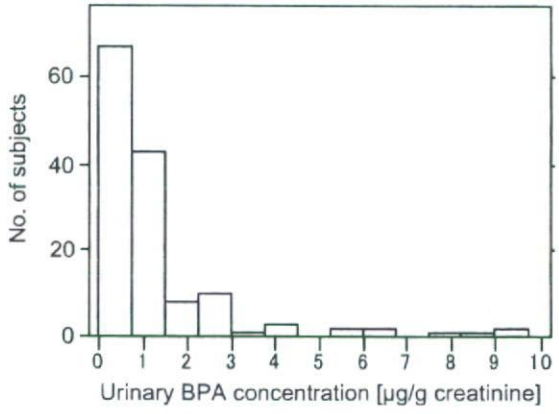

Fig. 1 Distribution of urinary BPA concentration (creatinine-adjusted; n=140).

Urinary concentrations of BPA in these women who consulted a physician for infertility were not higher than those in the previously surveyed populations. Our data are similar to those previously obtained at environmental concentrations in Japan (Table 1), albeit that these other studies used instrumental analyses for measurement, and some failed to consider BPA sulfate, although admittedly this is only a minor metabolite of BPA, in men at least (6). Specifically, our median concentrations (25th–75th percentiles) of unadjusted and creatinine-adjusted BPA of 1.6 (0.69–2.8) μg/L and 0.80 (0.45–1.3) μg/g creatinine, respectively, compare well with concentrations ranging from 0.1 to 11.9 (median of 0.77) μg/g creatinine in single spot urine samples collected from 48 female university students in Japan (8), and concentrations ranging from 0.14 to 5.47 (0.81 on average) μg/L in pooled urine samples in 46 male and 23 female volunteers (16).

For reference, one study showed median (25th–75th percentiles) BPA concentrations of 1.27 (0.49–2.46) μg/L and 1.77 (0.72–2.95) μg/g creatinine in urine from 210 women in the US (19), while another showed a median concentration (range) of 14.93 (0.0005–243.43) μg/g creatinine in urine from 160 Korean subjects and a geometric mean concentration of 5.01 μg/L in urine from 79 Korean females who were not

Table 1 Urinary BPA concentrations and endometriosis stages (n=140)

Endometriosis stage	No. of subjects	Urinary BPA concentration								
		Unadjusted [μg/L]			Creatinine-adjusted [μg/g creatinine]			Estimated daily intake [μg/kg body weight/day]*		
		Median	25th percentile	75th percentile	Median	25th percentile	75th percentile	Median	25th percentile	75th percentile
0–IV	140	1.57	0.69	2.78	0.80	0.45	1.29	0.016	0.010	0.026
0–I	81	1.32	0.63	2.40	0.74	0.45	1.21	0.014	0.009	0.025
II–IV	59	1.87	0.87	3.28	0.93	0.53	1.48	0.019	0.012	0.027
p for difference†				0.08			0.24			0.25

* n=131.

† Wilcoxon rank-sum test based on normal approximation.

occupationally exposed to BPA (20). Thus, our present values are similar to those obtained in other studies in Japan but not as high as those in Korean subjects.

In addition to BPA concentrations in spot urine, daily urinary BPA excretion rates based on 24-hour urine sampling has been measured (10, 16). Of interest, the rates of daily urinary BPA excretion are in direct accord with estimated values for daily BPA intake. The following values are also consistent with the daily BPA intake observed here (Table 1). The daily urinary excretion rates of BPA based on 24-hour urine samples collected from 36 male university students (mean age, 24.7 years) in 2003 ranged from <0.003 to 0.23 (median, 0.02) μg/kg body weight/day (10). Another study in which values were determined from whole-day urine samples from 11 male and 11 female volunteers showed daily urinary BPA excretion rates ranging from 0.48 to 4.5 (average, 1.68) μg/day (16). Furthermore, the observed range of estimated daily BPA intake (Table 1) was much lower than the tolerable daily intake of 50 μg/kg body weight/day established by the European Food Safety Authority in 2007 (21).

A change in BPA exposure over time has also been reported. Matsumoto et al. (22) measured BPA concentration in urine samples collected from university students in Japan and showed that the median of total BPA concentrations in 1992 was 2.2-fold higher than that in 1999. Furthermore, daily dietary BPA intake rates determined by measuring BPA concentrations in hospital-meal samples in Japan were in the range of 0.15–1.34 (average, 0.64) μg/day in 2000 but were only in the range of 0.06–0.68 (average, 0.20) μg/day in 2001 (23). Given the frequent detection of BPA in canned foods and retort foods (24), these reductions may have partly resulted from changes in the protective coating of epoxy on food and beverage cans (3). This finding may be useful in interpreting the meaning of urinary BPA levels in respective studies.

In addition to our exposure survey, we also explored whether urinary BPA concentration is associated with endometriosis in infertile Japanese women. Analysis of these data showed the absence of association. Although the pathogenesis of endometriosis is poorly understood, epidemiological evidence has suggested its association with several estrogen-dependent factors, namely early menarche, shorter menstrual cycle length, and lower parity (25–27). Based on this, endometriosis probably represents a sensitive detector of the effect of xenoestrogens in humans. However, two recent human experimental studies showing that orally administered BPA is quickly recovered in urine suggest that accurate exposure assessment based on urinary excretion is problematic, because it likely reflects recent rather than cumulative or long-term exposure (16, 17). The probable importance of chronic over recent exposure may partly explain our finding of the lack of association between BPA exposure and endometriosis, and future investigation should focus on the measurement of cumulative exposure to BPA.

Several other suggestions to improve studies of the association between BPA exposure and endometriosis can be made. In addition to accounting for cumulative exposure, investigation would be facilitated by a better study design, such as prospective cohort rather than cross-sectional studies. Measurement of free urinary BPA would be informative, providing its accuracy is confirmed, because BPA glucuronide shows no significant estrogenic activity, *in vitro* at least (2). Furthermore, a previous prospective cohort study showing that the risk of endometriosis is associated with exposure to diethylstilbestrol *in utero* indicates that early-life exposure to BPA, including fetal exposure, might also be critical to the development of endometriosis (28). Additionally, measurement of endogenous estrogen level would allow evaluation of the interaction between serum endogenous estrogens and BPA; this was not possible in the present study because individual endogenous estrogen level in individual premenopausal women periodically fluctuates in accordance with their menstrual cycle.

At a more basic concentration, studies of the effect of BPA on animals have also been inconclusive. BPA showed estrogenic activity generally 10^{-5}–10^{-3} times that of 17β-estradiol in a MCF-7 cell assay in a human breast cancer cell line (2). Furthermore, oral administration of BPA at 200 mg/kg body weight/day to a strain of immature rats resulted in an increase in uterine weight (2). In contrast, BPA showed no clear endocrine disrupting effect on rodents at estimated human exposure doses (5). Moreover, experimental results for BPA have been shown to depend on a number of factors, namely measurement endpoint, cell line, animal species and strain, and conjugate form and dose (2).

The strength of our study includes the use of a reliable biomarker measurement method and detailed information on subjects. In particular, we employed the most reliable analytical method currently available, namely, enzymatic deconjugation of both BPA sulfate and glucuronide and isotope-dilution LC-MS/MS measurement. Moreover, to monitor and control sample contamination, method blank tests were conducted in parallel with unknown sample analysis for all measurements, in addition to the rinsing of glassware and plastic tubing with methanol or acetonitrile before each test.

Two limitations of our study warrant mention. First, our subjects were restricted to urban residents of reproductive age, as well as other characteristics; thus, the generalizability of our results may also be limited. Second, intraindividual variation in BPA exposure and uncertainty in laboratory analysis may have contributed to urinary BPA concentration measurement errors. One study in which between-day variation in daily urinary BPA excretion was examined suggested that the magnitude of intraindividual variation is comparable to that in interindividual variation (10). If present, however, such errors would at least not tend to bias our results toward one side; thus, the median or geometric mean urinary BPA concentration would have been properly estimated. In contrast, interindividual variation, such as geometric standard deviation, might have been overestimated and thus remain to be corrected.

In conclusion, we report urinary concentrations of BPA in Japanese women who consulted a physician for infertility. Values were derived from the largest subject sample size studied in Japan to date and were obtained using the most reliable analytical method. Results showed that urinary BPA concentrations in these women were not higher than those in other populations. Moreover, this cross-sectional study revealed no association between urinary BPA concentration and endometriosis. Further study is required to confirm this result.

Appendix: Urinary BPA analysis

In the present study, we measured urinary bisphenol A (BPA) concentration. Here, we detail the methods used to measure urinary BPA concentration.

We analyzed urine samples without information on the participant's endometriosis status. Urinary BPA was separated, detected and measured using enzymatic deconjugation, offline solid-phase extraction, and high-performance liquid chromatography isotope-dilution tandem mass spectrometry (isotope-dilution LC-MS/MS). In addition to urinary BPA glucuronide, the recently identified compound urinary BPA sulfate has also been included in urinary BPA analysis in recent years (6–8, 29, 30). To deconjugate these BPAs into their free forms, we used β-glucuronidase/sulfatase (from *Helix pomatia* H-1, 492,000 units/g solid, Sigma Aldrich, St. Louis, MO, USA), a hydrolytic enzyme with sulfatase activity, as described in most recent studies (7, 29).

We purchased the native BPA standard (99.8%, for environmental analysis) and isotopically labeled standard (BPA-d_{16}, 99.9%, for environmental analysis) from Kanto Chemical Co., Inc. (Tokyo, Japan). Methanol (≥99.8%, residual-pesticide analysis grade 5000) and acetonitrile (≥99.8%, HPLC grade) were purchased from Sigma Aldrich Japan (Tokyo, Japan). Ammonium acetate (≥97.0%), sodium acetate trihydrate (≥99.0%), formic acid (≥98.0%), and acetic acid (≥99.7%) were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan). We used ultrapure water (Milli-Q Gradient A10, Millipore, Bedford, MA). To prepare 0.1 M acetate buffer, we diluted sodium acetate trihydrate (0.5 mmol) and acetic acid (0.5 mmol) with water to 10 ml. Standard substances were measured by weight and then dissolved in acetonitrile. They were serially diluted with acetonitrile:water=2:3 (v/v) to their respective target concentrations. Except for unused vials, spatula and glassware were previously rinsed with methanol three times and dried.

Frozen urine was thawed at room temperature. A 0.2-mL aliquot was decanted into a 2-mL silanized glass vial. We dissolved β-glucuronidase/sulfatase (0.2 mg) in 0.1 M acetate buffer solution (0.1 mL). This hydrolytic enzyme solution and 20 μL of the surrogate solution (50 μg/L) were added to the vial. This sample was mixed well and then incubated at 37°C overnight, as described in recent studies (7, 29).

A solid-phase extraction (SPE) cartridge (FOCUS Versa-Plate Tube, 10 mg/1.8 mL, Varian, Lake Forest, CA, USA) was preconditioned with 1-mL methanol followed by 1-mL water. After dilution with 1 M ammonium acetate aqueous solution (1 mL), the urine sample was vortex-mixed, and then loaded onto this SPE column. The SPE column was then washed with 1-mL water followed by acetonitrile:water=1:4 (v/v; 1 mL). Analytes were finally eluted from the SPE column using 1-mL solvent (methanol:acetonitorile:water:formic acid=6:3:1:0.01, v/v/v/v). A plastic tube (1.1 mL) into which the SPE eluate was collected was previously washed with acetonitrile. The eluate was dried out using a vacuum centrifuge (100 min) and the residue was reconstituted with acetonitrile:water=2:3 (v/v; 200 μL). The resulting solution was decanted into a silanized-glass insert.

We then measured BPA concentration in the extracted urine sample using a tandem quadrupole mass spectrometer (API 2000, Applied Biosystems, Foster City, CA, USA) connected to a high-performance liquid-chromatography (HPLC) system (LC-10 AD_{VP} pomp, SIL-10 AD_{VP} autosampler, CTO-10AC_{VP} column oven, and SCL-10A_{VP} system controller, Shimadzu, Kyoto, Japan) equipped with a C_{18} column (S-3 μm, 8 nm, 2.0 mm i.d.×100 mm, YMC-pack Pro RS, YMC Co., Ltd., Kyoto, Japan). We used 0.1-mL acetic acid in 1-L water (solvent A) and 0.1-mL acetic acid in 1-L acetonitrile (HPLC grade) (solvent B) as HPLC mobile phases with a constant flow rate (0.2 mL/min). A 20-μl aliquot of the sample was injected into the HPLC column using an auto sampler. The sample vial was cooled at 4.0°C. The percentage of solvent B was elevated from 40% (0 min) to 100% (5 min) for separation, kept at 100% (5–8 min) for flushing, and then kept at 40% (8.01–20 min) for re-equilibrium. The temperature of the HPLC column was kept constant (40.0°C). We used electrospray ionization and multiple-reaction monitoring to produce the combinations of the precursor and product ion of m/z 227.1 and 132.9 for BPA, and m/z 241.2 and 141.8 for BPA-d_{16}, respectively. The ion source temperature and collision energy were 500°C and −30 volts, respectively. MS/MS parameters on API 2000 were automatically optimized using a personal computer-based instrument software program (Analyst version 1.4, Applied Biosystems, Foster, CA, USA), which was also used to acquire and process the data obtained. To obtain a calibration curve (0.6–20 μg/L), each calibration point was weighted by the reciprocal of concentration (1/X), and the origin was ignored. In each analytical batch, one quality control material, all portions of which were obtained from a single sample of pooled urine (2.4 μg/L), and five method blanks were also analyzed along with unknown samples to ensure the accuracy of analysis. Intraday and interday reproducibilities (CV=8.8% and 19%, n=5, respectively) were previously checked by repeated measurements. In the batches where no method blank was observed, the standard deviation derived from six repeated measurements of a low-concentration standard solution (1 μg/L) was employed as the standard deviation at concentration zero (S_0). In the batch where method blanks were observed, S_0 was calculated from the standard deviation of the method blanks. We defined $3S_0$ as the analytical limits of detection (LODs), which were 0.30–0.55 μg/L. Mean total surrogate recovery was 72% in one batch (n=5). Any loss of BPA through analysis was automatically corrected on the basis of individual surrogate recovery. The mean of the method blank values was subtracted from the BPA measurement value in each batch.

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[Review]

Molecular Epidemiology and Urothelial Cancer

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Abstract : Tobacco smoking is the main cause of human urothelial cancer. It has been suggested that genetic susceptibility may contribute to the risk, because only a small portion of smokers develops urothelial cancer. Tobacco smoke contains many carcinogens which are activated or detoxified by phase-I or phase-II enzymes. The concentration of the ultimate carcinogen, which will react with DNA, is determined by the rate of activation and detoxification. Individuals with an increased rate of activation or a decreased rate of detoxification have a slightly higher level of bulky carcinogen-DNA adduct in the urothelial mucosa. Thus metabolic polymorphisms have been recognized as important determinants of carcinogen susceptibility, and recent efforts have shown that inter-individual differences in specific cytochrome P450 enzymes (CYPs), N-acetyltransferases (NAT), glutathione S-transferases (GST) and sulfotransferases (SULT) are often disproportionately represented in epidemiological studies between urothelial cancer cases and controls. It has been revealed that GSTM1 null genotype or NAT2 slow acetylator genotype may be associated with a small increase in urothelial cancer risk. Associations between other polymorphisms of metabolic enzymes and urothelial cancer are not well-known or are inconsistent. To reveal these associations, further well-designed and large-scale studies are needed.

Key words : molecular epidemiology, bladder cancer, cytochrome P450, glutathione S-transferase, N-acetyltransferase.

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Introduction

Many diseases are affected by both host factors and the external environment. In the case of urothelial cancer, the process of urothelial carcinogenesis is characterized by a diversity of risk factors. However, transitional cell urothelial cancer is the malignancy most causally linked to the process of chemical carcinogenesis. Extensive epidemiological studies have reported that 60−70% of bladder cancer cases can be attributed to exposure to certain chemical carcinogens [1, 2], the main sources of which are smoking and hazardous occupational exposure [2]. Tobacco smoke in particular is estimated to be responsible for 40−50% and 30% of all bladder cancer cases among males and females, respectively [1, 3, 4], whereas occupational exposure to chemicals, the oldest known causal factor in the development of bladder cancer, is now considered to be responsible for 19% of male and 6% of female cases [1, 3, 5]. There are 55 carcinogens in cigarette smoke that have been evaluated by the International Agency for Research on Cancer (IARC) and for which there is "sufficient evidence for carcinogenicity" in either laboratory animals or humans [6]. Polycyclic aromatic hydrocarbons (PAHs), aromatic amines, heterocyclic aromatic amines and *N*-nitroso compounds in tobacco smoke are thought to be urothelial carcinogens. However, many such compounds are not themselves carcinogenic but require activation by xenobiotic metabolizing enzymes. Many compounds are converted to reactive electrophilic metabolites by the oxidative (phase-I) enzymes, which are mainly cytochrome P450 enzymes (CYPs). Phase-II conjugating enzymes, such as glutathione *S*-transferases (GST), UDP-glucuronosyltransferases, sulfotransferases (SULT) and *N*-acetyltransferases (NAT), usually act as inactivation enzymes. Thus, the concerted action of these enzymes may be crucial in determining the final biological effects of a xenobiotic chemical. A number of genes that encode carcinogen-metabolizing enzymes are presently known. Individual variation in enzymes activating or detoxifying carcinogens and other xenobiotics have subsequently been related to discovered genetic polymorphisms in these genes. Epidemiological studies may contribute to our understanding of and may quantify the impact of xenobiotic metabolism on carcinogenesis in humans. This type of analysis is especially suitable for those enzymes that are polymorphically expressed on a heritable basis, enabling a comparison of cancer incidence among subjects with genetically deficient or extremely active metabolism to incidence among those with normal activity. Many molecular epidemiological studies showing an association between enzymatic polymorphisms and urothelial cancer susceptibility have been reported. Some enzymatic polymorphisms were associated with urothelial cancer susceptibility, but others were not associated with urothelial cancer susceptibility. There are reports with some significant association between enzymatic polymorphisms and urothelial cancer susceptibility related to cigarette smoking.

CYPs polymorphisms

1. *CYP1A1*

CYP1A1 is expressed in the lung, larynx, kidney, placenta, lymphocytes and fetal liver [7−9]. Substrates for and inducers of CYP1A1 include PAHs such as benzo[a]pyrene. PAHs

have been known as urothelial carcinogens from epidemiological and animal studies [10]. The CYP1A1-dependent phenotype has been determined through assay of the aryl hydrocarbon hydroxylase (AHH) metabolism of benzo[a]pyrene in human-derived tissues, usually peripheral blood lymphocytes [11]. Korsggard *et al.* [12] reported an association between AHH inducibility and urothelial cancer, and suggested that the role of AHH in urothelial carcinogenesis seemed to be less explicit (Table 1). Two genetic polymorphisms of the *CYP1A1* gene are thought to be associated with the large inter-individual differences in AHH enzyme activity. The *CYP1A1* Ile462Val polymorphism is a result of A (*CYP1A1*1A*) to G (*CYP1A1*2C*) substitution in exon 7, causing an amino acid change in the heme-binding region [13]. It has been reported that the Val/Val genotype resulted in a reduced catalytic enzyme activity [14]. The frequency of *CYP1A1* Val/Val genotype was reported to be 4.7－5.0% in Japanese populations and very rare in European populations [15]. The other *CYP1A1* polymorphism is a T (*CYP1A1**1A) to C (*CYP1A1**2A) transition 1197 bp downstream of exon 7, the MspI variant allele [16]. The MspI polymorphism can be classified into 3 genotypes: predominant homozygous alleles (genotype A), heterozygote (genotype B) and homozygous rare alleles (genotype C) [17]. The genotype C is closely related to high inducible CYP1A1 phenotypic activity [14]. Individuals with genotype C are most common among Chinese and Japanese (10%), and least common among Caucasians (0－4%), with African-Americans and Koreans (5－6%) [18]. The MspI polymorphism is closely linked to the *CYP1A1* Ile462Val polymorphism not only in a Japanese population but also in Northern Europeans.

There are two reports that showed a negative association between CYP1A1 genetic polymorphisms and urothelial cancer (Table 1) [19, 20]. Katoh *et al.* [19] reported that the frequency distribution of the *CYP1A1* Ile462Val genotypes in urothelial cancer patients showed no significant difference from that in healthy controls among Japanese populations. Brockmoller *et al.* [20] reported that polymorphisms in *CYP1A1* Ile462Val or MspI had no statistically significant impact in a German population. As the *CYP1A1* Val/Val genotype and genotype C are common among Asian populations but very rare among Europeans, the association between *CYP1A1* polymorphisms and urothelial cancer is interesting in Asian populations [21].

2. *CYP1A2*

CYP1A2 is involved in the metabolism of arylamines including 4-aminobiphenyl (ABP), nitrosamines and aromatic hydrocarbons, and the dehalogenation of chlorinated hydrocarbons. In humans, CYP1A2 has been detected only in the liver. A wide variation across racial/ethnic groups is one factor that may influence the results on the phenotypic distribution of slow, intermediate and rapid metabolizers of CYP1A2, as well as on the inducibility of this gene. Overall, slow CYP1A2 metabolizers are represented in about 10% of Caucasians, while their frequency in Japanese people seems to be much lower [22]. Evidence linking elevated CYP1A2 activity to increased bladder cancer risk has been reported [23, 24]. Lee *et al.* [24] reported the capacity for 3-demethylation of theophylline, as a reflection of CYP1A2 activity, was significantly associated with increased risk of non-occupational urinary bladder cancer (P=0.006). Moreover, molecular dosimetry studies indicate that the slow NAT2/rapid

Table 1. Previous reports of association between CYPs polymorphisms and urothelial cancer

Enzyme	Author, year of publication (reference number)	Population	Number of cases[1]	Number of controls	Phenotyping and genotyping (drug used for phenotyping or mutant allele)	Crude OR or result-urothelial cancer OR (95%CI)
CYP1A1	Korsgaard *et al*, 1984(12)	Swedish	76[2]	92	Phenotyping (3-methylcholanthrene)	1.42 (0.40−4.99) (renal pelvis and ureter) 1.38 (0.46−4.15) (bladder) (high/low+intermediate AHH ratio)
	Katoh *et al*, 1995(19)	Japanese	83[3]	101	Genotyping (*CYP1A1*2C*)	0.86 (0.47−1.54) (WM+MM/WW) 0.72 (0.17−3.11) (MM/WM+WW)
	Brockmoller *et al*, 1996(20)	German	374	373	Genotyping (*CYP1A1*2A,*2C*)	0.92 (0.61−1.41) (CYP1A1*2A WM+MM/WW) 0.67 (0.33−1.39) (CYP1A1*2C WM+MM/WW)
	Bringuier *et al*, 1998(21)	Australian	105[4]	0	Genotyping (*CYP1A1*2C*)	No association between p53 mutation and CYP1A1 polymorphism among cancer cases
CYP1A2	Lee *et al*, 1994(24)	Korean	100	84	Phenotyping (Theophylline)	A significant association between high CYP1A2 activity and bladder cancer risk(P=0.006)
CYP2A6	Tsukino *et al*, 2002(42)	Japanese	137[5]	92	Genotyping (*CYP2A6* deletion)	0.90 (0.26−3.14) (MM/WM+WW)
CYP2C19	Kaisary *et al*, 1987(44)	British	98	110	Phenotyping (Mephenytoin)	Not significant, but a weak association between non-aggressive bladder cancer and high CYP2C19 activity(P=0.04).
	Brockmoller *et al*, 1996(20)	German	374	373	Genotyping (*CYP2C19*2A*)	2.7 (0.9−7.7) (WM+WW/MM)
CYP2D6	Cartwright *et al*, 1984(49)	British	122	94	Phenotyping (Debrisoquine)	1.30 (0.09−18.28) (EM/PM) 0 WM or MM, P=0.04 among cases with past benzidine exposure (EM/PM)
	Kaisary *et al*, 1987(44)	British	98	110	Phenotyping (Debrisoquine)	Significant higher frequency of EM in aggressive cases(P=0.006), but not in non-aggressive cases
	Horai *et al*, 1989(50)	Japanese	51	203	Phenotyping (Metoprolol)	No significant association of WW status and cancer(P=1.0) No significantly different CYP2D6 frequency by gender, age, or tumor grade (EM/PM)
	Benites *et al*, 1990(51)	Spanish	125	556	Phenotyping (Debrisoquine)	2.6 (0.80−8.70) (EM/PM)
	Wolf *et al*, 1992(56)	British	184	720	Genotyping (*CYP2D6*4A*)	0.99 (0.45−2.19) (WW+WM/MM) 0.60 (0.43−0.83) (WW/WM+MM)
	Lee *et al*, 1994(24)	Korean	100	84	Phenotyping (Metoprolol/ α-hydroxymetoprolol)	0.59 (0.05−6.50) (EM/PM)
	Spurr *et al*, 1995(54)	British	126	132	Genotyping (*CYP2D6*4A*)	0.61 (0.22−1.68) (WW+WM/MM)
	Brockmoller *et al*, 1996(20)	German	374	373	Genotyping (*CYP2D6*2A*)	1.05 (0.57−1.95) (WW+WM/MM) 0.98 (0.74−1.31) (WW/WM+MM)
	Anwar *et al*, 1996(55)	Egyptian	22	21	Genotyping (*CYP2D6*3A, CYP2D6*4A,CYP2D6*5*)	2.36 (0.68−9.90) (WW+WM/MM)
	Chinegwundoh *et al*, 1996(57)	British	126	0	Genotyping (*CYP2D6*4A*)	No significantly different CYP2D6 frequency in smokers compared to nonsmokers(P=0.92).
CYP2E1	Anwar *et al*, 1996(55)	Egyptian	22	21	Genotyping (*CYP2E1*5B*)	Not significant(P=0.48)(0 MM or WM genotype among cases)
	Brockmoller *et al*, 1996(20)	German	374	373	Genotyping (*CYP2E1*1B,*5A,B*)	0.76 (0.54−1.08) (CYP2E1*1B WM+MM/WW) 1.16 (0.73−1.82) (CYP2E1*5A WM+MM/WW) 0.54 (0.27−1.08) (CYP2E1*5B WM+MM/WW)
	Farker *et al*, 1998(61)	German	273[6]	298	Genotyping (*CYP2E1*5A,B*)	1.14 (0.71−1.83) (CYP2E1*5A WM+MM/WW) 1.35 (0.65−2.78) (CYP2E1*5B WM+MM/WW)
	Farker *et al*, 2000(62)	German	158[6]	150	Genotyping (*CYP2E1*2*)	No variant allele among case and control patients
	Tsukino *et al*, 2002(42)	Japanese	137[5]	92	Genotyping(*CYP2E1*5B*)	0.67 (0.43−1.05) (WM+MM/WW)

[1]Cases were bladder cancer patients, [2]Cases were 46 bladder cancer and 30 renal pelvis cancer patients, [3]Cases were 65 bladder cancer,12 renal previs cancer and 6 ureter cancer patients, [4]Cases were renal pelvic cancer patients, [5]Cases were 95 bladder,14 renal pelvis, 16 ureter and 12 overlapping cancer patients, [6]Cases were renal and urothelial cancer patients

CYP1A2 individual has the highest level of ABP-hemoglobin adducts and, conversely, the lowest level of ABP-hemoglobin adducts, as observed in individuals who are rapid NAT2/slow CYP1A2 [23, 25]. Thus, those individuals who are rapid for CYP1A2 and slow for hepatic NAT2 might be at a higher risk for arylamine-induced urothelial cancer, compared with those who are slow for CYP1A2 and rapid for NAT2 [26]. Recently, several polymorphisms of *CYP1A2* have been reported [27−30], and two variant alleles which affect CYP1A2 activity were reported. One variant allele was a point mutation from guanine (*CYP1A2*1A*) to adenine (*CYP1A2*1C*) at position −2964 in the gene, which caused a significant decrease in CYP1A2 activity [28]. Another variant allele, a C to A transversion (*CYP1A2*1F*) in intron 1 at position 734 downstream of the first transcribed nucleotide, has recently been associated with increased CYP1A2 inducibility [29]. The outcome of these polymorphisms on susceptibility to urothelial cancer is not known.

3. *CYP2A6*

CYP2A6 is a constitutive cytochrome P450 which is expressed in human liver at variable levels [31]. This enzyme may also be expressed in other tissues, including nasal tissue, but not in the kidney, duodenum, lung, alveolar macrophages, peripheral lymphocytes, placenta or uterine endometrium [32]. CYP2A6 catalyzes the metabolic activation of several pre-carcinogens, including several nitrosamines, aflatoxin B1 and 1,3-butadiene. In addition, CYP2A6 is involved in the metabolism of nicotine, the primary compound in tobacco that establishes and maintains tobacco dependence [33, 34]. Individual variation in the activity of this enzyme has been suggested to be linked to differential smoking behaviours [35]. A large inter-individual difference in CYP2A6-mediated coumarin 7-hydroxylase activity [36, 37] suggested the existence of a genetic polymorphism of this enzyme. Several variants of the *CYP2A6* gene have been identified, and recently, several deletion-type alleles of the *CYP2A6* (*CYP2A6*4*) have been identified [38-41]. The homozygous deletion *CYP2A6* frequency of 3.2−4.0% was found only in Oriental populations [42]. The homozygous deletion *CYP2A6* genotype causes complete lack of enzymatic activity. Seventy to eighty percent of nicotine is converted to cotinine, mainly by CYP2A6, and the proportion of urinary cotinine excretion in the individuals with the homozygous deletion of *CYP2A6* was about one-seventh compared to the *CYP2A6* wild types [41]. It has been hypothesized that a lack of CYP2A6 activity might decrease the production of genotoxic metabolites of these nitrosamines and potentially reduces the risk of tobacco-smoking related cancer by this mechanism. Our study revealed the frequency of the homozygous deletion of *CYP2A6* genotype was 2.9% (4/137) in the urothelial patients, compared with 3.2% (7/210) in the controls (OR=0.84, 95% CI=0.24−2.96) [42]. However, the sample size of our study was small, and future research is needed to establish a significant relationship.

4. *CYP2C19*

The human CYP2Cs metabolizes approximately 20% of clinically used drugs, and there are four members in the subfamily: CYP2C8, CYP2C9, CYP2C19 and CYP2C18. The most well-known of CYP2Cs' polymorphism is in CYP2C19. Several defective CYP2C19 alleles are the