cancer. These results suggest that the effects of isoflavone may differ according to stage. One mechanism by which isoflavones reduce the risk of prostate cancer seems to involve estrogen receptor β in prostate tissue (31), but cancer with higher metastatic potential is associated with the complete or partial loss of estrogen receptor β expression (32-34). Moreover, animal studies in rats showed that the beneficial effects of a soy diet play a role in the early stages of tumor development but have no effect in invasive prostate cancer (35, 36). On this basis, isoflavones may prevent the early stages of prostate cancer development only. Clinically significant localized prostate cancer likely arises from latent cancer and then develop to advanced cancer with high mortality (4, 37). Given that the incidence of latent prostate cancer in Japanese men is the same as in Western men despite a lower incidence of prostate cancer (1, 4, 5), isoflavone may delay the progression of latent prostate cancer.

When we limited analysis to men ages >60 years, the association between isoflavone and localized prostate cancer was strengthened. Hoffman et al. (38) reported that men with cancers detected by prostate-specific antigen screening were more often younger than those men in whom cancer was clinically diagnosed. Our study also showed that the proportion of screening-detected cancers was higher (54.6%) in those men ages ≤60 years than in those ages >60 years (28.1%), although prostate-specific antigen screening information was available for only 70% of subjects. However, although we analyzed the association between localized prostate cancer and isoflavones after excluding screening-detected tumors, results did not change. Isoflavone may be protective for localized prostate cancer only in men ages >60 years and may not have a protective effect in the early stage of prostate cancer in younger men.

Our study has several methodologic strengths. First, it was a prospective design, which diminishes the probability of recall bias that is inherent to case-control studies. Second, we evaluated isoflavone intake using a validated questionnaire, and participants had a large variation in isoflavone consumption. One reason for the inconsistent findings for the association between soy food and prostate cancer in previous studies may be errors in exposure measurements and the small exposure variation in Western subjects. Third, we adjusted possible confounding factors to remove associations with other substances. It is also possible that a lifestyle associated with a high intake of soy food may have contributed to the risk of prostate cancer. In this study, the associations between isoflavones and prostate cancer were strengthened after

Table 3. RRs and 95% CIs for prostate cancer according to quartile of energy-adjusted intake of genistein, daidzein, miso soup, and soy food by stage

	Intake by quartile				P_{trend}
	Lowest	Second	Third	Highest	
Localized prostate cancer					
Genistein					
No. cases	54	51	73	42	
Person-years of follow-up	78,329	81,340	83,086	82,219	
Age/area–adjusted RR (95% CI)	1.00	0.81 (0.55-1.20)	1.21 (0.84-1.74)	0.67 (0.44-1.03)	0.31
Multivariate RR (95% CI)	1.00	0.77 (0.52-1.15)	1.16 (0.79-1.69)	0.59 (0.38-0.93)	0.15
Daidzein					
No. cases	51	54	71	44	
Person-years of follow-up	78,150	81,441	83,686	82,296	
Age/area–adjusted RR (95% CI)	1.00	0.90 (0.61-1.32)	1.22 (0.84-1.77)	0.75 (0.49-1.15)	0.50
Multivariate RR (95% CI)	1.00	0.86 (0.57-1.28)	1.20 (0.82-1.76)	0.66 (0.42-1.04)	0.27
Miso soup					
No. cases	44	55	65	56	
Person-years of follow-up	75,540	79,526	84,287	85,620	
Age/area–adjusted RR (95% CI)	1.00	0.96 (0.65-1.43)	0.99 (0.67-1.46)	0.83 (0.55-1.25)	0.40
Multivariate RR (95% CI)	1.00	0.95 (0.63-1.43)	0.98 (0.65-1.46)	0.78 (0.51-1.20)	0.29
Soy food					
No. cases	46	64	58	52	
Person-years of follow-up	77,644	81,454	83,017	82,857	
Age/area–adjusted RR (95% CI)	1.00	1.17 (0.80-1.72)	1.05 (0.70-1.55)	0.88 (0.58-1.32)	0.38
Multivariate RR (95% CI)	1.00	1.06 (0.71-1.57)	0.93 (0.61-1.40)	0.77 (0.50-1.19)	0.17
Advanced prostate cancer					
Genistein					
No. cases	16	23	15	20	
Person-years of follow-up	78,416	81,440	83,193	82,264	
Age/area–adjusted RR (95% CI)	1.00	1.46 (0.77-2.78)	1.02 (0.49-2.11)	1.32 (0.65-2.67)	0.69
Multivariate RR (95% CI)	1.00	1.60 (0.78-3.30)	1.11 (0.50-2.48)	1.26 (0.56-2.83)	0.88
Daidzein					
No. cases	14	23	18	19	
Person-years of follow-up	78,243	81,537	83,185	82,347	
Age/area–adjusted RR (95% CI)	1.00	1.66 (0.85-3.25)	1.40 (0.68-2.88)	1.47 (0.70-3.09)	0.45
Multivariate RR (95% CI)	1.00	1.67 (0.79-3.52)	1.39 (0.63-3.10)	1.43 (0.63-3.28)	0.58
Miso soup					
No. cases	11	18	18	27	
Person-years of follow-up	75,639	79,620	84,382	85,671	
Age/area–adjusted RR (95% CI)	1.00	1.51 (0.70-3.23)	1.58 (0.72-3.44)	2.21 (1.05-4.66)	0.04
Multivariate RR (95% CI)	1.00	1.73 (0.73-4.12)	1.65 (0.67-4.04)	2.79 (1.19-6.55)	0.02
Soy food					
No. cases	16	20	19	19	
Person-years of follow-up	77,736	81,557	83,096	82,923	
Age/area–adjusted RR (95% CI)	1.00	1.21 (0.62-2.35)	1.15 (0.58-2.27)	1.07 (0.53-2.15)	0.92
Multivariate RR (95% CI)	1.00	1.36 (0.65-2.85)	1.19 (0.55-2.56)	1.05 (0.47-2.34)	0.92

NOTE: Multivariate RRs were adjusted for age, area, smoking status, drinking frequency, marital status, body mass index, and intake of total fatty acids, dairy, vegetables, and fruits.

Table 4. RRs and 95% CIs for prostate cancer according to quartile of energy-adjusted intake of genistein, daidzein, miso soup, and soy food by stage categorized according to age

	Intake in quartile				P_{trend}
	Lowest	Second	Third	Highest	
>60 y					
Localized prostate cancer					
Genistein					
No. cases	42	37	38	27	
Person-years of follow-up	24,531	25,312	25,859	25,538	
Multivariate RR (95% CI)	1.00	0.81 (0.51-1.29)	0.79 (0.49-1.28)	0.52 (0.30-0.90)	0.03
Daidzein					
No. cases	41	36	42	25	
Person-years of follow-up	24,475	25,398	25,790	25,576	
Multivariate RR (95% CI)	1.00	0.80 (0.50-1.28)	0.90 (0.56-1.45)	0.50 (0.28-0.88)	0.04
Miso soup					
No. cases	34	32	44	34	
Person-years of follow-up	23,842	24,855	26,264	26,279	
Multivariate RR (95% CI)	1.00	0.72 (0.43-1.18)	0.87 (0.54-1.41)	0.65 (0.39-1.11)	0.22
Soy food					
No. cases	35	47	37	25	
Person-years of follow-up	24,270	25,488	25,866	25,616	
Multivariate RR (95% CI)	1.00	1.12 (0.71-1.79)	0.76 (0.46-1.27)	0.52 (0.29-0.90)	0.01
Advanced prostate cancer					
Genistein					
No. cases	15	15	11	12	
Person-years of follow-up	24,585	25,381	25,924	25,572	
Multivariate RR (95% CI)	1.00	1.16 (0.51-2.64)	0.82 (0.33-2.07)	1.03 (0.41-2.59)	0.87
Daidzein					
No. cases	11	19	11	12	
Person-years of follow-up	24,536	25,465	25,852	25,611	
Multivariate RR (95% CI)	1.00	1.97 (0.83-4.66)	1.16 (0.43-3.12)	1.49 (0.55-4.03)	0.75
Miso soup					
No. cases	9	15	13	16	
Person-years of follow-up	23,915	24,904	26,314	26,329	
Multivariate RR (95% CI)	1.00	2.01 (0.74-5.45)	1.79 (0.63-5.13)	2.86 (1.01-8.11)	0.07
Soy food					
No. cases	14	18	11	10	
Person-years of follow-up	24,332	25,539	25,933	25,659	
Multivariate RR (95% CI)	1.00	1.49 (0.66-3.34)	1.06 (0.43-2.54)	0.70 (0.25-1.95)	0.38
≦60 y					
Localized prostate cancer					
Genistein					
No. cases	14	19	24	19	
Person-years of follow-up	53,645	55,739	57,270	57,080	
Multivariate RR (95% CI)	1.00	1.10 (0.55-2.22)	1.51 (0.76-2.98)	1.18 (0.57-2.45)	0.49
Daidzein					
No. cases	14	21	20	21	
Person-years of follow-up	53,555	55,728	57,310	57,310	
Multivariate RR (95% CI)	1.00	1.17 (0.58-2.39)	1.44 (0.71-2.93)	1.38 (0.67-2.83)	0.32
Miso soup					
No. cases	14	20	24	18	
Person-years of follow-up	51,470	54,766	57,743	59,756	
Multivariate RR (95% CI)	1.00	0.96 (0.48-1.92)	1.02 (0.52-2.00)	0.82 (0.40-1.68)	0.62
Soy food					
No. cases	13	20	22	21	
Person-years of follow-up	52,997	55,845	57,205	57,687	
Multivariate RR (95% CI)	1.00	1.17 (0.58-2.39)	1.44 (0.71-2.93)	1.38 (0.67-2.83)	0.32
Advanced prostate cancer					
Genistein					
No. cases	4	5	3	9	
Person-years of follow-up	53,692	55,772	57,296	57,102	
Multivariate RR (95% CI)	1.00	1.05 (0.28-4.00)	0.44 (0.08-2.53)	2.00 (0.53-7.51)	0.32
Daidzein					
No. cases	3	5	5	8	
Person-years of follow-up	53,598	55,772	57,339	57,153	
Multivariate RR (95% CI)	1.00	1.42 (0.33-6.05)	1.21 (0.26-5.75)	2.46 (0.57-10.60)	0.23
Miso soup					
No. cases	2	4	6	9	
Person-years of follow-up	51,506	54,800	57,775	59,781	
Multivariate RR (95% CI)	1.00	1.66 (0.30-9.14)	1.78 (0.34-9.43)	2.65 (0.54-12.89)	0.20
Soy food					
No. cases	3	5	6	7	
Person-years of follow-up	53,044	55,871	57,239	57,708	
Multivariate RR (95% CI)	1.00	1.27 (0.30-5.42)	1.15 (0.26-5.05)	1.48 (0.35-6.20)	0.63

NOTE: Multivariate RRs were adjusted for age, area, smoking status, drinking frequency, marital status, body mass index, and intake of total fatty acids, dairy, vegetables, and fruits.

Table 5. RRs of prostate cancer according to quartile of energy-adjusted intake of genistein, daidzein, miso soup, and soy food after excluding screening-detected tumors by stage in men aged more than 60 years old

	Intake in quartile				P_{trend}
	Lowest	Second	Third	Highest	
>60 y					
Localized prostate cancer					
Genistein					
No. cases	32	42	23	17	
Person-years of follow-up	24,457	25,225	25,766	25,455	
Multivariate RR (95% CI)	1.00	0.73 (0.41-1.29)	0.71 (0.39-1.29)	0.52 (0.27-1.03)	0.07
Daidzein					
No. cases	31	22	28	15	
Person-years of follow-up	24,401	25,313	25,699	25,490	
Multivariate RR (95% CI)	1.00	0.69 (0.39-1.24)	0.92 (0.52-1.61)	0.49 (0.24-1.00)	0.13
Miso soup					
No. cases	25	17	30	24	
Person-years of follow-up	23,766	24,780	26,160	26,196	
Multivariate RR (95% CI)	1.00	0.58 (0.30-1.10)	0.95 (0.54-1.70)	0.73 (0.39-1.38)	0.67
Soy food					
No. cases	25	29	26	16	
Person-years of follow-up	24,199	25,383	25,782	25,538	
Multivariate RR (95% CI)	1.00	1.02 (0.58-1.81)	0.79 (0.43-1.46)	0.51 (0.26-1.01)	0.04
Advanced prostate cancer					
Genistein					
No. cases	18	14	10	11	
Person-years of follow-up	24,501	25,276	25,826	25,474	
Multivariate RR (95% CI)	1.00	0.97 (0.46-2.03)	0.62 (0.25-1.50)	0.85 (0.35-2.05)	0.50
Daidzein					
No. cases	13	19	11	10	
Person-years of follow-up	24,449	25,360	25,759	25,509	
Multivariate RR (95% CI)	1.00	1.69 (0.79-3.63)	0.96 (0.39-2.39)	1.10 (0.43-2.87)	0.85
Miso soup					
No. cases	12	14	10	17	
Person-years of follow-up	23,817	24,819	26,218	26,224	
Multivariate RR (95% CI)	1.00	1.32 (0.55-3.16)	1.10 (0.43-2.84)	1.97 (0.80-4.86)	0.18
Soy food					
No. cases	14	20	9	10	
Person-years of follow-up	24,246	25,427	25,835	25,569	
Multivariate RR (95% CI)	1.00	1.82 (0.86-3.86)	0.92 (0.37-2.30)	0.73 (0.27-2.00)	0.31

NOTE: Multivariate RRs were adjusted for age, area, smoking status, drinking frequency, marital status, body mass index, and intake of total fatty acids, dairy, vegetables, and fruits.

adjustment for several confounding factors. Fourth, response rate was high (~80%), and the proportion of subjects lost to follow-up was relatively low (0.1%).

On the other hand, the present study had several limitations. One was our inability to distinguish screening-detected cancer from total prostate cancer. It is possible that men who have health check-ups are more health conscious and may consume more soy food. However, such misclassification, if present, would lead to increase the risk of localized prostate cancer. Therefore, this inability to distinguish would not account for the decreased risk of localized prostate cancer. Another limitation was that the number of advanced prostate cancer cases was small. A larger sample size may have detected the positive effects of isoflavones on advanced prostate cancer with greater precision. Moreover, misclassification of exposure due to changes in isoflavone consumption during the study period might have occurred because we used information on consumption obtained at one point only. If present, however, such misclassification would underestimate the true relative risk.

In summary, we found that isoflavone intake was associated with a decreased risk of localized prostate cancer but tended to be associated with an increased risk of advanced prostate cancer. Recent interest has focused on whether isoflavones have chemopreventive effects. Given that Japanese consume isoflavones regularly throughout life, we do not yet know the period during which the effects of isoflavones on prostate cancer are preventive. Further research is required, including well-designed clinical trials in humans.

Appendix A

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References

1. Parkin DM, Whelan SL, Ferlay J, Thomas DB. Cancer incidence in five continents. Vol. VIII. Lyon: IARC; 2002.
2. Tsugane S, de Souza JM, Costa ML, Jr., et al. Cancer incidence rates among Japanese immigrants in the city of Sao Paulo, Brazil, 1969-78. Cancer Causes Control 1990;1:189–93.
3. Shimizu H, Ross RK, Bernstein L, Yatani R, Henderson BE, Mack TM. Cancers of the prostate and breast among Japanese and white immigrants in Los Angeles County. Br J Cancer 1991;63:963–6.
4. Yatani R, Kusano I, Shiraishi T, Hayashi T, Stemmermann GN. Latent prostatic carcinoma: pathological and epidemiological aspects. Jpn J Clin Oncol 1989;19:319–26.
5. Muir CS, Nectoux J, Staszewski J. The epidemiology of prostatic cancer. Geographical distribution and time-trends. Acta Oncol 1991;30:133–40.
6. Magee PJ, Rowland IR. Phyto-oestrogens, their mechanism of action: current evidence for a role in breast and prostate cancer. Br J Nutr 2004;91:513–31.
7. Morton MS, Arisaka O, Miyake N, Morgan LD, Evans BA. Phytoestrogen concentrations in serum from Japanese men and women over forty years of age. J Nutr 2002;132:3168–71.
8. Adlercreutz H, Markkanen H, Watanabe S. Plasma concentrations of phyto-oestrogens in Japanese men. Lancet 1993;342:1209–10.
9. Morton MS, Chan PS, Cheng C, et al. Lignans and isoflavonoids in plasma and prostatic fluid in men: samples from Portugal, Hong Kong, and the United Kingdom. Prostate 1997;32:122–8.
10. Jacobsen BK, Knutsen SF, Fraser GE. Does high soy milk intake reduce prostate cancer incidence? The Adventist Health Study (United States). Cancer Causes Control 1998;9:553–7.
11. Strom SS, Yamamura Y, Duphorne CM, et al. Phytoestrogen intake and prostate cancer: a case-control study using a new database. Nutr Cancer 1999;33:20–5.
12. Kolonel LN, Hankin JH, Whittemore AS, et al. Vegetables, fruits, legumes and prostate cancer: a multiethnic case-control study. Cancer Epidemiol Biomarkers Prev 2000;9:795–804.
13. Lee MM, Gomez SL, Chang JS, Wey M, Wang RT, Hsing AW. Soy and isoflavone consumption in relation to prostate cancer risk in China. Cancer Epidemiol Biomarkers Prev 2003;12:665–8.
14. Allen NE, Sauvaget C, Roddam AW, et al. A prospective study of diet and prostate cancer in Japanese men. Cancer Causes Control 2004;15:911–20.
15. Jian L, Zhang DH, Lee AH, Binns CW. Do preserved foods increase prostate cancer risk? Br J Cancer 2004;90:1792–5.
16. Nomura AM, Hankin JH, Lee J, Stemmermann GN. Cohort study of tofu intake and prostate cancer: no apparent association. Cancer Epidemiol Biomarkers Prev 2004;13:2277–9.
17. Sonoda T, Nagata Y, Mori M, et al. A case-control study of diet and prostate cancer in Japan: possible protective effect of traditional Japanese diet. Cancer Sci 2004;95:238–42.
18. Otani T, Iwasaki M, Inoue M, Tsugane S. Body mass index, body height, and subsequent risk of colorectal cancer in middle-aged and elderly Japanese men and women: Japan public health center-based prospective study. Cancer Causes Control 2005;16:839–50.
19. Kimira M, Arai Y, Shimoi K, Watanabe S. Japanese intake of flavonoids and isoflavonoids from foods. J Epidemiol 1998;8:168–75.
20. Arai Y, Watanabe S, Kimira M, Shimoi K, Mochizuki R, Kinae N. Dietary intakes of flavonols, flavones and isoflavones by Japanese women and the inverse correlation between quercetin intake and plasma LDL cholesterol concentration. J Nutr 2000;130:2243–50.
21. Yamamoto S, Sobue T, Sasaki S, et al. Validity and reproducibility of a self-administered food-frequency questionnaire to assess isoflavone intake in a Japanese population in comparison with dietary records and blood and urine isoflavones. J Nutr 2001;131:2741–7.
22. Willett W. Nutritional epidemiology. 2nd ed. New York (NY): Oxford University Press; 1998.
23. Ishihara J, Sobue T, Yamamoto S, et al. Validity and reproducibility of a self-administered food frequency questionnaire in the JPHC Study Cohort II: study design, participant profile and results in comparison with Cohort I. J Epidemiol 2003;13:S134–47.
24. Sasaki S, Ishihara J, Tsugane S. Reproducibility of a self-administered food frequency questionnaire used in the 5-year follow-up survey of the JPHC Study Cohort I to assess food and nutrient intake. J Epidemiol 2003;13: S115–24.
25. Tsugane S, Kobayashi M, Sasaki S. Validity of the self-administered food frequency questionnaire used in the 5-year follow-up survey of the JPHC Study Cohort I: comparison with dietary records for main nutrients. J Epidemiol 2003;13:S51–6.
26. WHO. International classification of diseases for oncology. 3rd ed. Geneva (Switzerland): WHO; 2000.
27. Parkin DM, Chen VW, Ferlay J, Galceran J, Storm HH, Whelan SL. Comparability and quality control in cancer registration. IARC Technical Report No. 19. Lyon: IARC; 1994.
28. Nagata C, Inaba S, Kawakami N, Kakizoe T, Shimizu H. Inverse association of soy product intake with serum androgen and estrogen concentrations in Japanese men. Nutr Cancer 2000;36:14–8.
29. Holzbeierlein JM, McIntosh J, Thrasher JB. The role of soy phytoestrogens in prostate cancer. Curr Opin Urol 2005;15:17–22.
30. Evans BA, Griffiths K, Morton MS. Inhibition of 5 α-reductase in genital skin fibroblasts and prostate tissue by dietary lignans and isoflavonoids. J Endocrinol 1995;147:295–302.
31. Ganry O. Phytoestrogens and prostate cancer risk. Prev Med 2005;41:1–6.
32. Horvath LG, Henshall SM, Lee CS, et al. Frequent loss of estrogen receptor-β expression in prostate cancer. Cancer Res 2001;61:5331–5.
33. Leav I, Lau KM, Adams JY, et al. Comparative studies of the estrogen receptors β and α and the androgen receptor in normal human prostate glands, dysplasia, and in primary and metastatic carcinoma. Am J Pathol 2001;159:79–92.
34. Fixemer T, Remberger K, Bonkhoff H. Differential expression of the estrogen receptor β (ERβ) in human prostate tissue, premalignant changes, and in primary, metastatic, and recurrent prostatic adenocarcinoma. Prostate 2003; 54:79–87.
35. Landstrom M, Zhang JX, Hallmans G, et al. Inhibitory effects of soy and rye diets on the development of Dunning R3327 prostate adenocarcinoma in rats. Prostate 1998;36:151–61.
36. Bylund A, Zhang JX, Bergh A, et al. Rye bran and soy protein delay growth and increase apoptosis of human LNCaP prostate adenocarcinoma in nude mice. Prostate 2000;42:304–14.
37. Vij U, Kumar A. Phyto-oestrogens and prostatic growth. Natl Med J India 2004;17:22–6.
38. Hoffman RM, Stone SN, Espey D, Potosky AL. Differences between men with screening-detected versus clinically diagnosed prostate cancers in the USA. BMC Cancer 2005;5:27.

Effect of Soy Isoflavones on Endometriosis

Interaction With Estrogen Receptor 2 Gene Polymorphism

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Background: Progression of endometriosis is considered estrogen-dependent. Dietary soy isoflavones may affect the risk of endometriosis, and polymorphisms in estrogen receptor genes may modify this association. We examined associations among soy isoflavone intake, estrogen receptor 2 (*ESR2*) gene polymorphisms and risk of endometriosis.

Methods: We recruited women age 20–45 years old who had consulted a university hospital for infertility in Tokyo, Japan in 1999 or 2000. A total of 138 eligible women were diagnosed laparoscopically and classified into 3 subgroups: control (no endometriosis), early endometriosis (stage I–II) and advanced endometriosis (stage III–IV). We measured urinary levels of genistein and daidzein as markers for dietary intake of soy isoflavones, and genotyped *ESR2* gene *Rsa*I polymorphisms.

Results: Higher levels of urinary genistein and daidzein were associated with decreased risk of advanced endometriosis (P for trend = 0.01 and 0.06, respectively) but not early endometriosis. For advanced endometriosis, the adjusted odds ratio for the highest quartile group was 0.21 (95% confidence interval = 0.06–0.76) for genistein and 0.29 (0.08–1.03) for daidzein, when compared with the lowest group. Inverse associations were also noted between urinary isoflavones and the severity of endometriosis (P for trend = 0.01 for genistein and 0.07 for daidzein). For advanced endometriosis, *ESR2* gene *Rsa*I polymorphism appeared to modify the effects of genistein (P for interaction = 0.03).

Conclusions: Dietary isoflavones may reduce the risk of endometriosis among Japanese women.

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Soy isoflavones are phytoestrogens found in soybeans. Phytoestrogens are plant-derived nonsteroidal compounds that possess estrogen-like biologic activities. These compounds reportedly display weak estrogenic and antiestrogenic properties.[1–3] The 2 primary isoflavones found in soy are genistein and daidzein. Structural similarities allow isoflavones to bind to estrogen receptors.[4]

It has been hypothesized that soy isoflavones may play a role in the etiology of estrogen-related diseases and several epidemiologic studies have been conducted; however, findings have been complicated and inconsistent.[5–7] A prospective study in Japan, where isoflavone intake is known to be relatively high, showed a protective effect on postmenopausal breast cancer.[5] On the other hand, a nested case–control study in the United Kingdom, where intake is relatively low, showed that serum and urinary isoflavone levels were associated with increased breast cancer risk.[6] A recent meta-analysis found a small reduction in breast cancer risk associated with soy intake.[7] However, the authors suggested that the results should be interpreted cautiously due to potential exposure misclassification, confounding, lack of a dose-response pattern and the possibility of adverse effects of soy constituents.

Endometriosis is a benign, proliferative disease in which tissue similar to endometrial tissue is found outside the uterus—usually in the pelvic cavity, but sometimes in distant organs. Endometriosis is commonly accompanied by pelvic pain and infertility. Both genetic and environmental factors may contribute.[8] The reported prevalence of largely asymptomatic endometriosis found in women undergoing tubal ligation is about 4%, ranging from 1% to 7%.[9] Progression of endometriosis is considered estrogen-dependent.[10] Soy isoflavones might thus be expected to affect the risk and severity of endometriosis. However, few studies have investigated the effects of soy isoflavones on endometriosis.

Several studies have recently described associations between estrogen receptor (ESR) gene polymorphisms and endometriosis.[11–13] Genistein and daidzein reportedly display much greater affinity for ESR2 than for ESR1,[14] suggesting

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that the estrogenic or antiestrogenic properties of soy isoflavones may occur preferentially through ESR2. Although functional variability of *ESR2* gene polymorphisms could feasibly be associated with response to soy isoflavones, whether *ESR2* gene polymorphisms exert altered phenotypic effects on endometriosis through interactions with soy isoflavones is not known.

The present study investigated whether urinary genistein and daidzein are associated with risk and severity of endometriosis, and whether polymorphisms in the *ESR2* gene are associated with response to soy isoflavones.

METHODS

Study Protocol and Ethics

This study was part of a case–control study conducted on a Japanese population to investigate associations between genetic and environmental factors in endometriosis.[15] We recruited consecutive female patients age 20 to 45-year-old who attended the Department of Obstetrics and Gynecology at Jikei University School of Medicine Hospital for infertility in 1999 or 2000. 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 therapy before laparoscopic examination.

All study protocols were approved by the Institutional Review Boards of Jikei University, National Cancer Center and National Institute for Environmental Studies. 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 to collect information on demographic factors, age, height, weight, medical history for themselves and their families, reproductive and menstrual history, oral contraceptive use, food- and alcohol-consumption frequency, and smoking history.

Participants collected first morning urine sample using a paper cup and plastic tube, and gave a fasting blood sample before the laparoscopic examination. Blood samples were divided into plasma and buffy layers. All biologic samples were stored at −80°C until analysis.

Diagnosis of Endometriosis

Laparoscopy is necessary for definitive diagnosis of endometriosis. In the present study, all participants underwent diagnostic laparoscopy, and stage of endometriosis was determined by trained gynecologists in accordance with the revised classifications of the American Fertility Society.[16] 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.[17] Considering the more severe stages as a separate category thus appears logical.[18] Based on surgically or pathologically confirmed disease status, we classified cases into 2 subgroups: early (Stage I–II) or advanced endometriosis (stage III–IV). Women without endometriosis were defined as controls.

Determination of Soy Isoflavone Levels

Urinary levels of soy isoflavones offer a useful biomarker for dietary intake and plasma concentration of isoflavones.[19–21] The present study measured urinary levels of genistein and daidzein as markers for dietary intake of soy isoflavones. A total of 30 mL of first-morning urine was collected before laparoscopic examination. Genistein and daidzein levels were analyzed using high-performance liquid chromatography with a coulometric array detector in accordance with the modified methods of Gamache and Acworth.[22]

Concentrations of genistein and daidzein were determined by linear regression of peak height for each standard, and were adjusted according to recovery rate of the internal standard. The regression coefficient of peak height and concentration calculated for soy isoflavones revealed a linearity range of 0–8.0 μg/mL, with correlation coefficient values >0.995. Voltametric response for the standard solution displayed coefficients of variation of 2.7%–8.4% for intraday variation and 11.1%–12.2% for interday variation. Recovery rates of soy isoflavones in urine samples ranged between approximately 85% and 100%. Detection limits were 3.22 ng/mL for genistein and 4.14 ng/mL for daidzein.

Concentrations of urinary genistein and daidzein were adjusted by urinary creatinine concentration to correct for variability in urine dilution (μmol/g Cre). All measurements were performed by investigators blinded to case–control status.

Genotyping of *ESR2* Gene Polymorphism

The *ESR2 Rsa*I polymorphism, comprising a G-to-A change at nucleotide 1082 in exon 5, was genotyped using polymerase chain reaction (PCR) restriction fragment length polymorphism methods.[23] Blood samples were obtained before laparoscopic examination. Genomic DNA samples were extracted from peripheral white blood cells using a standard protease K method. PCR products were digested using 5 U of *Rsa*I restriction enzyme at 37°C for 8 hours, then electrophoresed on a 3% agarose gel containing ethidium bromide.

In this study, *ESR2 Rsa*I polymorphism is represented by the *r* and *R* alleles, with *R* indicating the presence of corresponding restriction sites, and *r* indicating the absence of restriction sites. For quality control, blinded control samples were inserted to validate genotyping identification procedures. Concordance for blinded samples was 100%. Genotyping was conducted by investigators blinded to case–control status.

Statistical Analysis

To assess differences between cases and controls, basic characteristics and possible risk factors for endometriosis were compared using Student *t* test and the χ^2 test. Spearman correlation coefficients between urinary level of genistein and daidzein were calculated. We calculated odds ratios (ORs) and 95% confidence intervals (CIs) for urinary levels by an unconditional logistic regression model following classification into medians or quartiles based on control distribution. Linear trends for ORs were tested in the unconditional logistic regression model by treating the categories as ordinal variables. We evaluated trends for median values according to disease stage to assess associations between urinary levels of genistein and daidzein and disease stage.

To compare observed and expected genotype frequencies, we tested for Hardy-Weinberg equilibrium by using an exact test. *ESR2 Rsa*I polymorphism was classified into 2 subgroups according to the presence of corresponding restriction sites: *r/r* genotype; and *R/r* + *R/R* genotype. ORs and 95% CIs were calculated for associations between *ESR2 Rsa*I polymorphism and endometriosis using the unconditional logistic regression model.

To investigate whether the *ESR2 Rsa*I genotype modified the effect of urinary levels of genistein or daidzein, we calculated ORs and 95% CIs of endometriosis according to a combination of subgroups for the *ESR2 Rsa*I genotype and urinary isoflavones, using the unconditional logistic regression model. A low level of urinary isoflavones in combination with *R/r* + *R/R* genotype was considered as the reference group. Interactions between *ESR2 Rsa*I polymorphism and urinary isoflavones in the risk of endometriosis were tested with the Wald test using product terms between urinary genistein or daidzein and genotypes.

The present study was designed to have 80% power to detect a decrease in risk of two-thirds at the 5% level of significance. All statistical tests were based on 2-tailed probabilities. We adjusted ORs and 95% CIs for possible confounding factors of endometriosis, namely age (continuous), menstrual cycle (continuous), and duration of menstrual bleeding (less than 7 days or 7 days or more).[9,10] We used SPSS for Windows software version 11.0 (SPSS JAPAN, Tokyo, Japan) for statistical analyses.

RESULTS

Baseline Characteristics and Possible Risk Factors for Endometriosis

Table 1 shows baseline characteristics and possible risk factors for endometriosis in controls and cases. No important differences in mean age or body mass index were identified between groups. Distribution of menstrual bleeding, hypermenorrhea, and smoking also did not differ substantially. The advanced endometriosis group had a shorter mean menstrual cycle length than controls (controls, 30.7 ± 6.1 days; advanced endometriosis, 28.3 ± 3.0 days) and was more likely to have menstrual cramps and dyspareunia.

TABLE 1. Baseline Characteristics and Possible Risk Factors for Endometriosis

Baseline Characteristics	Controls (n = 59)	Endometriosis	
		Early (Stage I–II) (n = 31)	Advanced (Stage III–IV) (n = 48)
Age (yrs); mean ± SD	33.1 ± 4.1	32.3 ± 3.2	32.6 ± 3.7
Body mass index (kg/m^2); mean ± SD	21.0 ± 3.4	20.6 ± 2.1	20.2 ± 2.1
Menstrual cycle (d); mean ± SD	30.7 ± 6.1	29.6 ± 3.6	28.3 ± 3.0
Menstrual bleeding; no. (%)			
<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. (%)			
No	39 (66)	20 (65)	29 (60)
Yes	18 (31)	7 (23)	15 (31)
Missing	2 (3)	4 (13)	4 (8)
Menstrual cramps; no. (%)			
No	10 (17)	2 (6)	1 (2)
Yes	47 (80)	25 (81)	44 (92)
Missing	2 (3)	4 (13)	3 (6)
Dyspareunia; no. (%)			
No	31 (53)	12 (39)	10 (21)
Yes	25 (42)	14 (45)	34 (71)
Missing	3 (5)	5 (16)	4 (8)
Smoking status; no. (%)			
Never	38 (64)	19 (61)	29 (60)
Current or ever-smoker	19 (32)	8 (26)	15 (31)
Missing	2 (3)	4 (13)	4 (8)

Effect of Urinary Isoflavones on Endometriosis

Table 2 shows risk of endometriosis according to median or quartile levels of urinary isoflavones. In controls, median isoflavone level was 3.24 μmol/g Cre for genistein and 4.01 μmol/g Cre for daidzein. The Spearman correlation coefficient between genistein and daidzein was 0.84. Urinary genistein and daidzein levels were inversely associated with advanced endometriosis (*P* for trend = 0.01 and 0.06, respectively) but not with early endometriosis. For advanced endometriosis, the adjusted odds ratio for the highest quartile group was 0.21 (95% CI = 0.06–0.76) for genistein and 0.29 (0.08–1.03) for daidzein when compared with the lowest group.

Table 3 shows the trends of median values for urinary isoflavones according to disease stage. An inverse relationship with stage of endometriosis was observed for both genistein levels (*P* for trend = 0.01) and daidzein levels (*P* for trend = 0.07).

Associations Between *ESR2 Rsa*I Polymorphism and Endometriosis

Table 4 shows the genotypic distribution of *ESR2 Rsa*I polymorphism and associations with risk of endometriosis. The *ESR2 Rsa*I *r/r* genotype was predominant. Allele fre-

TABLE 2. Association between Urinary Isoflavone Level and Risk of Endometriosis

Urinary Isoflavone	No. Controls	Endometriosis: Early (Stage I–II) No.	Early (Stage I–II) OR (95% CI)*	Endometriosis: Advanced (Stage III–IV) No.	Advanced (Stage III–IV) ORs (95% CI)*
Genistein (μmol/g creatinine)					
<1.60†	14	7	1.00	22	1.00
1.60–3.23	15	12	1.86 (0.49–7.09)	13	0.65 (0.21–2.01)
3.24–6.49	15	7	0.82 (0.18–3.80)	7	0.40 (0.12–1.34)
≥6.50	15	5	0.63 (0.14–2.89)	6	0.21 (0.06–0.76)
P for trend			0.34		0.01
Low level (<3.24)†	29	19	1.00	35	1.00
High level (≥3.24)	30	12	0.50 (0.18–1.39)	13	0.35 (0.14–0.87)
Daidzein (μmol/g creatinine)					
<1.94†	14	5	1.00	16	1.00
1.94–4.00	15	7	1.87 (0.41–8.57)	15	0.84 (0.26–2.73)
4.01–7.94	15	12	2.16 (0.49–9.41)	10	0.65 (0.20–2.09)
≥7.95	15	7	1.33 (0.30–5.97)	7	0.29 (0.08–1.03)
P for trend			0.73		0.06
Low level (<4.01)†	29	12	1.00	31	1.00
High level (≥4.01)	30	19	1.21 (0.45–3.27)	17	0.49 (0.21–1.15)

*Adjusted for age (continuous); menstrual cycle (continuous); and duration of menstrual bleeding (less than 7 days or 7 days or more).
†Reference category.

TABLE 3. Median Values† of Urinary Isoflavone Level and Stage of Endometriosis

Urinary Isoflavone	Controls (n = 59)	Early (Stage I–II) (n = 31)	Advanced (Stage III–IV) (n = 48)	*P* for Trend*
Genistein (μmol/g creatinine)	3.2 (1.6–6.5)	2.6 (1.7–5.2)	1.7 (0.6–4.1)	0.01
Daidzein (μmol/g creatinine)	4.0 (1.9–8.0)	4.9 (2.6–7.6)	2.6 (1.0–5.0)	0.07

*Jonckheere-Terpstra test.
†Median (25th–75th percentile).

quencies of *ESR2 Rsa*I polymorphism were 0.77 for the *r* allele and 0.23 for the *R* allele. In addition, the distribution of *ESR2 Rsa*I polymorphism was in Hardy–Weinberg equilibrium ($P = 0.26$). The *ESR2 Rsa*I *r/r* genotype was associated with reduced risk of early endometriosis compared with the *R/r* + *R/R* genotype (OR = 0.30; CI = 0.11–0.85). The association was weaker for advanced endometriosis (0.67; 0.29–1.55).

TABLE 4. Association Between *ESR2 Rsa*I Polymorphism and Risk of Endometriosis

Genotype*	No. Controls	Early (Stage I–II) No.	Early (Stage I–II) OR (95% CI)†	Advanced (Stage III–IV) No.	Advanced (Stage III–IV) OR (95% CI)†
R/r + *R/R*‡	26	21	1.00	26	1.00
r/r	33	10	0.30 (0.11–0.85)	22	0.67 (0.29–1.55)

*Exact test for Hardy–Weinberg equilibrium: $P = 0.26$.
†Adjusted for age (continuous); menstrual cycle (continuous); and duration of menstrual bleeding (less than 7 days or 7 days or more).
‡Reference category.

Interactions Between *ESR2 Rsa*I Polymorphism and Urinary Isoflavones in the Risk of Endometriosis

Table 5 shows ORs and 95% CIs of endometriosis for combinations of *ESR2 Rsa*I genotype and urinary isoflavone levels. Compared with subjects with the *ESR2 Rsa*I *R/r* + *R/R* genotype and a low genistein level, ORs of advanced endometriosis were lower among the 3 other groups. The adjusted OR was 0.10 (95% CI = 0.02–0.48) for subjects with *ESR2 Rsa*I *R/r* + *R/R* genotype with high genistein level; 0.32 (0.10–1.04) for subjects with *ESR2 Rsa*I *r/r* genotype with low genistein level; 0.27 (0.08–0.92) for subjects with *ESR2 Rsa*I *r/r* genotype with high genistein

TABLE 5. Interactions Between ESR2 RsaI Polymorphism and Urinary Isoflavone in the Risk of Endometriosis

Genotype	Urinary Isoflavone (μmol/g creatinine)	No. Controls	Early (Stage I–II)		Advanced (Stage III–IV)	
			No.	OR (95%CI)*	No.	OR (95%CI)*
	Genistein					
R/r + *R/R*	Low†	11	11	1.00	22	1.00
R/r + *R/R*	High	15	10	0.88 (0.23–3.38)	4	0.10 (0.02–0.48)
r/r	Low	18	8	0.54 (0.15–1.93)	3	0.32 (0.10–1.04)
r/r	High	15	2	NC	9	0.27 (0.08–0.92)
P for interaction				NC		0.03
	Daidzein					
R/r + *R/R*	Low†	15	10	1.00	19	1.00
R/r + *R/R*	High	11	11	1.28 (0.33–4.96)	7	0.35 (0.09–1.34)
r/r	Low	14	2	0.18 (0.03–1.09)	12	0.56 (0.18–1.78)
r/r	High	19	8	0.44 (0.12–1.61)	10	0.39 (0.13–1.20)
P for interaction				0.58		0.45

*Adjusted for age (continuous); menstrual cycle (continuous); and duration of menstrual bleeding (less than 7 days or 7 days or more).
†Reference category.
NC, estimates were not calculated due to missing data.

level. A significant interaction was noted between *ESR2 Rsa*I polymorphism and genistein levels in risk of advanced endometriosis (*P* for interaction = 0.03). Interactions between *ESR2 Rsa*I polymorphism and genistein level were not observed in early endometriosis. Although a similar pattern was observed for ORs of both early and advanced endometriosis for the combinations of *ESR2 Rsa*I genotype and urinary daidzein level, these may have been due to chance.

DISCUSSION

The present study showed an inverse association between urinary isoflavones and the risk of advanced endometriosis. This association was stronger for genistein than daidzein. In addition, there was statistical evidence for interaction between urinary genistein and *ESR2* gene polymorphisms.

The reduced risk of endometriosis following ingestion of soy isoflavones may be attributable to antiestrogenic properties of these compounds. A previous study showed that prolonged exposure to genistein results in decreased levels of estrogen receptor mRNA in addition to decreased response to estradiol stimulation.[24] Plasma levels of isoflavones can be 10,000- to 100,000-fold higher than those of estradiol.[25] When the relative binding affinity of 17β-estradiol was set at 100 in solid-phase competition experiments, relative binding affinity for ESR2 was 87 for genistein and 0.5 for daidzein.[14] Although the elimination half-life from blood and urine is reportedly 7–8 hours for both genistein and daidzein,[26] long-term soy diets may modify the physiologic effects of estrogens. Given these facts, a lower prevalence of endometriosis might be expected in Japanese populations compared with Western countries, as with breast cancer. Nevertheless, the prevalence of endometriosis in the Japanese general population remains unclear due to the need for surgical diagnosis.

Our finding showed that the strength of association was stronger for genistein than for daidzein. One possible explanation is the difference in their binding affinities to ESR2. A second possibility is based on the difference in metabolism between genistein and daidzein. Daidzein can be metabolized to equol and *O*-desmethylangolites by intestinal bacteria, and these metabolites are absorbed, enter the circulation, and are excreted in urine. Although equol has been suggested to possess stronger estrogenic properties than genistein, some individuals are capable of equol production whereas others are not, probably because of differences in gut microflora. This difference might play a role in the weaker associations for daidzein than genistein.[27]

ESR2 plays important roles in endometrial function, in addition to the well-known role of ESR1 in endometrial proliferation and differentiation.[28] The *ESR2 Rsa*I polymorphism does not cause amino acid changes, but may well be associated with altered ligand-binding affinity or transcriptional activity. Genes containing single nucleotide polymorphisms (SNPs) can cause different structural folds in mRNA,[29] and these mRNA variants may possess different biologic functions during interactions with other cellular components. Altered estrogen or soy isoflavone signal transduction thanks to *ESR2* gene polymorphisms may be directly responsible for interindividual susceptibility to and severity of endometriosis.

The present study found evidence of an interaction between urinary genistein and *ESR2* gene polymorphisms. Isoflavones may play a more effective role among the *ESR2 Rsa*I *R/r* + *R/R* genotype than the *r/r* genotype, although the latter itself is likely to be protective for endometriosis. This result should be interpreted cautiously, however, because of the relatively small number of subjects—a major limitation of

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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REFERENCES

1. Tham DM, Gardner CD, Haskell WL. Clinical review 97: potential health benefits of dietary phytoestrogens: a review of the clinical, epidemiological, and mechanistic evidence. *J Clin Endocrinol Metab*. 1998;83:2223–2235.
2. Wagner JD, Anthony MS, Cline JM. Soy phytoestrogens: research on benefits and risks. *Clin Obstet Gynecol*. 2001;44:843–852.
3. Gruber CJ, Tschugguel W, Schneeberger C, et al. Production and actions of estrogens. *N Engl J Med*. 2002;346:340–352.
4. Limer JL, Speirs V. Phyto-oestrogens and breast cancer chemoprevention. *Breast Cancer Res*. 2004;6:119–127.
5. Yamamoto S, Sobue T, Kobayashi M, et al. Soy, isoflavones, and breast cancer risk in Japan. *J Natl Cancer Inst*. 2003;95:906–913.
6. Grace PB, Taylor JI, Low YL, et al. Phytoestrogen concentrations in serum and spot urine as biomarkers for dietary phytoestrogen intake and their relation to breast cancer risk in European prospective investigation of cancer and nutrition-norfolk. *Cancer Epidemiol Biomarkers Prev*. 2004;13:698–708.
7. Trock BJ, Hilakivi-Clarke L, Clarke R. Meta-analysis of soy intake and breast cancer risk. *J Natl Cancer Inst*. 2006;98:459–471.
8. Kennedy S. The genetics of endometriosis. *J Reprod Med*. 1998;43:263–268.
9. Cramer DW, Missmer SA. The epidemiology of endometriosis. *Ann NY Acad Sci*. 2002;955:11–22.
10. Vigano P, Parazzini F, Somigliana E, et al. Endometriosis: epidemiology and aetiological factors. *Best Pract Res Clin Obstet Gynaecol*. 2004;18:177–200.
11. Georgiou I, Syrrou M, Bouba I, et al. Association of estrogen receptor gene polymorphisms with endometriosis. *Fertil Steril*. 1999;72:164–166.
12. Kitawaki J, Obayashi H, Ishihara H, et al. Oestrogen receptor-alpha gene polymorphism is associated with endometriosis, adenomyosis and leiomyomata. *Hum Reprod*. 2001;16:51–55.
13. Wang Z, Yoshida S, Negoro K, et al. Polymorphisms in the estrogen receptor beta gene but not estrogen receptor alpha gene affect the risk of developing endometriosis in a Japanese population. *Fertil Steril*. 2004;81:1650–1656.
14. Kuiper GG, Lemmen JG, Carlsson B, et al. Interaction of estrogenic chemicals and phytoestrogens with estrogen receptor beta. *Endocrinology*. 1998;139:4252–4263.
15. Tsukino H, Hanaoka T, Sasaki H, et al. Associations between serum levels of selected organochlorine compounds and endometriosis in infertile Japanese women. *Environ Res*. 2005;99:118–125.
16. American Fertility Society. Revised American Fertility Society classification of endometriosis. *Fertil Steril*. 1985;43:351–352.
17. Marchino GL, Gennarelli G, Enria R, et al. Diagnosis of pelvic endometriosis with use of macroscopic versus histologic findings. *Fertil Steril*. 2005;84:12–15.
18. Zondervan KT, Cardon LR, Kennedy SH. What makes a good case-control study? Design issues for complex traits such as endometriosis. *Hum Reprod*. 2002;17:1415–1423.
19. Maskarinec G, Singh S, Meng L, et al. Dietary soy intake and urinary isoflavone excretion among women from a multiethnic population. *Cancer Epidemiol Biomarkers Prev*. 1998;7:613–619.
20. Arai Y, Uehara M, Sato Y, et al. Comparison of isoflavones among dietary intake, plasma concentration and urinary excretion for accurate estimation of phytoestrogen intake. *J Epidemiol*. 2000;10:127–135.
21. Yamamoto S, Sobue T, Sasaki S, et al. Validity and reproducibility of a self-administered food-frequency questionnaire to assess isoflavone intake in a japanese population in comparison with dietary records and blood and urine isoflavones. *J Nutr*. 2001;131:2741–2747.
22. Gamache PH, Acworth IN. Analysis of phytoestrogens and polyphenols in plasma, tissue, and urine using HPLC with coulometric array detection. *Proc Soc Exp Biol Med*. 1998;217:274–280.
23. Sundarrajan C, Liao WX, Roy AC, et al. Association between estrogen receptor-beta gene polymorphisms and ovulatory dysfunctions in patients with menstrual disorders. *J Clin Endocrinol Metab*. 2001;86:135–139.
24. Wang TT, Sathyamoorthy N, Phang JM. Molecular effects of genistein on estrogen receptor mediated pathways. *Carcinogenesis*. 1996;17:271–275.
25. Manach C, Williamson G, Morand C, et al. Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. *Am J Clin Nutr*. 2005;81(1 Suppl):230S–242S.

26. King RA, Bursill DB. Plasma and urinary kinetics of the isoflavones daidzein and genistein after a single soy meal in humans. *Am J Clin Nutr*. 1998;67:867–872.
27. Atkinson C, Frankenfeld CL, Lampe JW. Gut bacterial metabolism of the soy isoflavone daidzein: exploring the relevance to human health. *Exp Biol Med (Maywood)*. 2005;230:155–170.
28. Lecce G, Meduri G, Ancelin M, et al. Presence of estrogen receptor beta in the human endometrium through the cycle: expression in glandular, stromal, and vascular cells. *J Clin Endocrinol Metab*. 2001;86:1379–1386.
29. Shen LX, Basilion JP, Stanton VP Jr. Single-nucleotide polymorphisms can cause different structural folds of mRNA. *Proc Natl Acad Sci USA*. 1999;96:7871–876.
30. Missmer SA, Hankinson SE, Spiegelman D, et al. Reproductive history and endometriosis among premenopausal women. *Obstet Gynecol*. 2004;104:965–974.
31. Missmer SA, Hankinson SE, Spiegelman D, et al. Incidence of laparoscopically confirmed endometriosis by demographic, anthropometric, and lifestyle factors. *Am J Epidemiol*. 2004;160:784–796.

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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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References

American Fertility Society. Revised American Fertility Society classification of endometriosis. *Fertil Steril* 1985;**43**:351–352.

Brennan P. Gene–environment interaction and aetiology of cancer: what does it mean and how can we measure it? *Carcinogenesis* 2002;**23**:381–387.

Eskenazi B, Mocarelli P, Warner M *et al.* Serum dioxin concentrations and endometriosis: a cohort study in Seveso, Italy. *Environ Health Perspect* 2002;**110**:629–634.

Gonzalez FJ. The molecular biology of cytochrome P450s. *Pharmacol Rev* 1988;**40**:243–288.

Gottardis MM, Robinson SP, Satyaswaroop PG *et al.* Contrasting actions of tamoxifen on endometrial and breast tumor growth in the athymic mouse. *Cancer Res* 1988;**48**:812–815.

Heilier JF, Nackers F, Verougstraete V *et al.* Increased dioxin-like compounds in the serum of women with peritoneal endometriosis and deep endometriotic (adenomyotic) nodules. *Fertil Steril* 2005;**84**:305–312.

Huang CS, Shen CY, Chang KJ *et al.* Cytochrome P4501A1 polymorphism as a susceptibility factor for breast cancer in postmenopausal Chinese women in Taiwan. *Br J Cancer* 1999;**80**:1838–1843.

Kennedy S. The genetics of endometriosis. *J Reprod Med* 1998;**43**:263–268.

Laden F, Ishibe N, Hankinson SE *et al.* Polychlorinated biphenyls, cytochrome P450 1A1, and breast cancer risk in the Nurses' Health Study. *Cancer Epidemiol Biomarkers Prev* 2002;**11**:1560–1565.

Landi MT, Bergen AW, Baccarelli A *et al.* CYP1A1 and CYP1B1 genotypes, haplotypes, and TCDD-induced gene expression in subjects from Seveso, Italy. Toxicology 2005;**207**:191–202.

Lebel G, Dodin S, Ayotte P *et al.* Organochlorine exposure and the risk of endometriosis. *Fertil Steril* 1998;**69**:221–228.

Li Y, Millikan RC, Bell DA *et al.* Polychlorinated biphenyls, cytochrome P450 1A1 (CYP1A1) polymorphisms, and breast cancer risk among African American women and white women in North Carolina: a population-based case-control study. *Breast Cancer Res* 2005;**7**:R12–R18.

Louis GM, Weiner JM, Whitcomb BW *et al.* Environmental PCB exposure and risk of endometriosis. *Hum Reprod* 2005;**20**:279–285.

Marchino GL, Gennarelli G, Enria R *et al.* Diagnosis of pelvic endometriosis with use of macroscopic versus histologic findings. *Fertil Steril* 2005;**84**:12–15.

Martucci CP, Fishman J. P450 enzymes of estrogen metabolism. *Pharmacol Ther* 1993;**57**:237–257.

Mayani A, Barel S, Soback S *et al.* Dioxin concentrations in women with endometriosis. *Hum Reprod* 1997;**12**:373–375.

Missmer SA, Hankinson SE, Spiegelman D *et al.* Incidence of laparoscopically confirmed endometriosis by demographic, anthropometric, and lifestyle factors. *Am J Epidemiol* 2004a;**160**:784–796.

Missmer SA, Hankinson SE, Spiegelman D *et al.* Reproductive history and endometriosis among premenopausal women. *Obstet Gynecol* 2004b;**104**:965–974.

Moysich KB, Shields PG, Freudenheim JL *et al.* Polychlorinated biphenyls, cytochrome P4501A1 polymorphism, and postmenopausal breast cancer risk. *Cancer Epidemiol Biomarkers Prev* 1999;**8**:41–44.

Ohtake F, Takeyama K, Matsumoto T *et al.* Modulation of oestrogen receptor signalling by association with the activated dioxin receptor. *Nature* 2003;**423**:545–550.

Pauwels A, Schepens PJ, D'Hooghe T *et al.* The risk of endometriosis and exposure to dioxins and polychlorinated biphenyls: a case–control study of infertile women. *Hum Reprod* 2001;**16**:2050–2055.

Rawson JM. Prevalence of endometriosis in asymptomatic women. *J Reprod Med* 1991;**36**:513–515.

Signorello LB, Harlow BL, Cramer DW *et al.* Epidemiologic determinants of endometriosis: a hospital-based case-control study. *Ann Epidemiol* 1997;**7**:267–271.

Solus JF, Arietta BJ, Harris JR *et al.* Genetic variation in eleven phase I drug metabolism genes in an ethnically diverse population. *Pharmacogenomics* 2004;**5**:895–931.

Tang YM, Green BL, Chen GF *et al.* Human CYP1B1 Leu432Val gene polymorphism: ethnic distribution in African-Americans, Caucasians and Chinese; oestradiol hydroxylase activity; and distribution in prostate cancer cases and controls. *Pharmacogenetics* 2000;**10**:761–766.

Toppari J, Larsen JC, Christiansen P *et al.* Male reproductive health and environmental xenoestrogens. *Environ Health Perspect* 1996;**104**: 741–803.

Treloar SA, O'Connor DT, O'Connor VM *et al.* Genetic influences on endometriosis in an Australian twin sample. *Fertil Steril* 1999;**71**: 701–710.

Tsukino H, Hanaoka T, Sasaki H *et al.* Associations between serum levels of selected organochlorine compounds and endometriosis in infertile Japanese women. *Environ Res* 2005;**99**:118–125.

Van den Berg M, Birnbaum L, Bosveld AT *et al.* Toxic equivalency factors (TEFs) for PCBs, PCDDs, PCDFs for humans and wildlife. *Environ Health Perspect* 1998;**106**:775–792.

Whysner J, Williams GM. 2,3,7,8-Tetrachlorodibenzo-p-dioxin mechanistic data and risk assessment: gene regulation, cytotoxicity, enhanced cell proliferation, and tumor promotion. *Pharmacol Ther* 1996;**71**: 193–223.

Yang JZ, Agarwal SK, Foster WG. Subchronic exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin modulates the pathophysiology of endometriosis in the cynomolgus monkey. *Toxicol Sci* 2000;**56**:374–381.

Yang Q, Khoury MJ, Flanders WD. Sample size requirements in case-only designs to detect gene-environment interaction. *Am J Epidemiol* 1997;**146**:713–720.

Zhang Y, Wise JP, Holford TR *et al.* Serum polychlorinated biphenyls, cytochrome P-450 1A1 polymorphisms, and risk of breast cancer in Connecticut women. *Am J Epidemiol* 2004;**160**:1177–1183.

Zondervan KT, Cardon LR, Kennedy SH. What makes a good case-control study? Design issues for complex traits such as endometriosis. *Hum Reprod* 2002;**17**:1415–1423.

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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.

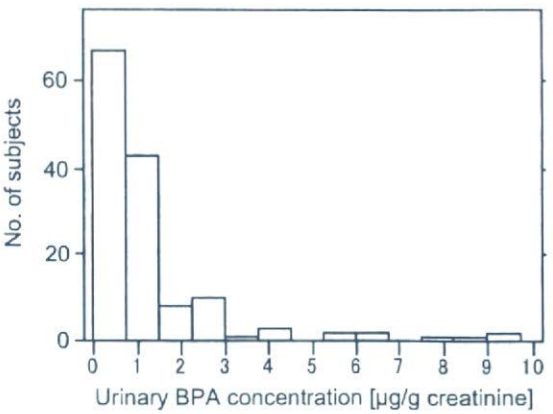

Fig. 1 Distribution of urinary BPA concentration (creatinine-adjusted; n=140).

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.