women (83%) returned the questionnaire, which contained questions concerning their demographic characteristics, medical history, menstrual and reproductive history, anthropometric factors, physical activity, smoking and drinking habits, and diet.

2.3. Blood collection

A total of 10 ml blood was provided voluntarily by subjects during their health checkups in 1990–1995. Blood samples were divided into plasma and buffy layers and then stored at −80 °C until analysis. Among respondents to the baseline questionnaire, a total of 24,996 women (45%) donated blood.

2.4. Follow-up

All registered subjects were followed from the start of the study period to December 31, 2002. Data on residential relocation were obtained from residential registries. Among study subjects (n=24,996), 1289 subjects (5.2%) moved out of the study area and 5 (0.02%) were lost to follow-up within the study at-risk period.

2.5. Selection of cases and controls

Incidence data on breast cancer for the JPHC cancer registry were collected via two data sources, major local hospitals and population-based cancer registries. Death certificates were used to supplement the information on cancer incidence. Site of origin and histologic type were coded by members of our Study Group using the International Classification of Diseases for Oncology, Third Edition (ICD-O-3), code: C500-509. Hormone receptor status was determined in a relatively large number of clinical laboratories, primarily using enzyme-linked immunoassay rather than immunohistochemical techniques. Hormone receptor positivity values were determined either as specified by the laboratory that performed the assay, or in accordance with the laboratory's written interpretation thereof, or both. Up to the end of the study period, 144 new breast cancer cases were identified from among the 24,226 women who had returned the baseline questionnaire, did not report a history of breast cancer or ovarian cystoma, and provided blood samples. Diagnosis was microscopically verified in 98% of cases. The mortality/incidence ratio was 0.14 and the proportion of cases for which information was available from death certificates only was 0.7%.

For each case, two controls were selected from subjects who had no prior history of breast cancer when the case was diagnosed. Controls were matched for each case by age (within 3 years), PHC area, area (city or town and village), date of blood collection (within 90 days), time of day of blood collection (within 3 hours), fasting time at blood collection (within 3 hours) and menopausal status at blood collection.

2.6. Laboratory assay

The plasma organochlorines measured were p,p'-dichlorodiphenyltrichloroethane (p,p'-DDT), p,p'-DDE, hexachlorobenzene (HCB), and β-hexachlorocyclohexane (β-HCH) following extraction from a 500-ul plasma sample according to the method of Gill et al. (Gill et al., 1996). Quantitative analysis was undertaken on a gas-chromatography isotope-dilution mass-spectrometry (GC-IDMS) (QP2010, Shimadzu Co., Kyoto, Japan) under the following operating conditions for GC: methyl silicon capillary column (DB-1, 10 m×0.1 mm ID, 0.1 um film thickness, J&W Scientific, USA); carrier gas, helium with a flow rate of 0.16 ml/min; splitless injection mode (1 ul); inlet temperature, 250 °C; column oven temperature program, 70 °C

Table 1 – Characteristics of cases and matched control subjects at baseline

	Cases (n=139)		Controls (n=278)		p [a]
Age (year), mean (SE)	51.8	(0.60)	51.9	(0.43)	–
Family history of breast cancer, n (%)	2	(1.4)	2	(0.7)	0.48
Premenopausal women, n (%)	55	(41)	110	(41)	–
Age at menopause (year), mean (SE) [b]	50.0	(0.39)	49.8	(0.28)	0.71
Age at menarche (year), mean (SE) [b]	14.6	(0.15)	14.8	(0.10)	0.33
Number of births, mean (SE) [b]	2.3	(0.12)	2.8	(0.09)	0.01
Age at first birth (year), mean (SE) [b]	25.7	(0.30)	25.0	(0.22)	0.29
Use of exogenous female hormones (current use), n (%)	3	(2.3)	2	(0.8)	0.22
Height (cm), mean (SE) [b]	151.7	(0.47)	151.3	(0.34)	0.67
Body mass index (kg/m^2), mean (SE) [b]	23.4	(0.26)	23.6	(0.18)	0.64
Smoking (current smoker), n (%)	5	(3.6)	15	(5.4)	0.30
Alcohol drinking (regular drinker), n (%)	17	(12)	24	(8.7)	0.33
Leisure-time physical activity (≥once per week), n (%)	28	(21)	53	(19)	0.42
Vitamin supplement user, n (%)	31	(23)	61	(23)	0.87
Total energy intake (kcal/day), mean (SE) [c]	1812.0	(12.0)	1804.6	(8.5)	0.60
Fish and shellfish intake (g/day), mean (SE) [c]	109.7	(1.2)	109.8	(0.82)	0.83
Meat intake (g/day), mean (SE) [c]	68.5	(0.52)	67.8	(0.37)	0.11
Vegetable intake (g/day), mean (SE) [c]	296.7	(1.1)	295.7	(0.78)	0.16
Fruit intake (g/day), mean (SE) [c]	181.3	(2.1)	179.4	(1.5)	0.78

[a] p for Mantel–Haenszel test with matched-pair strata.
[b] Adjusted for age.
[c] Intake for each subject was estimated from the food frequency questionnaires based on a regression function derived from the validation study data.

Table 2 – Crude plasma organochlorine levels in cases and matched control subjects

	Cases (n=139)		Controls (n=278)		p[a]
	Median	(interquartile range)	Median	(interquartile range)	
p,p'-DDT (ng/ml)[b]	1.10	(0.64, 2.04)	1.12	(0.72, 1.83)	0.19
p,p'-DDE (ng/ml)[b]	7.04	(4.36, 10.42)	6.08	(3.72, 9.70)	0.42
HCB (ng/ml)[b]	0.29	(0.15, 0.51)	0.29	(0.18, 0.51)	0.73
β-HCH (ng/ml)[b]	0.51	(0.22, 1.10)	0.50	(Not detected, 0.94)	0.77

[a] p for Mantel–Haenszel test with matched-pair strata.
[b] p,p'-DDT, dichlorodiphenyltrichloroethane; p,p'-DDE, dichlorodiphenyldichloroethylene; HCB, hexachlorobenzene; and β-HCH, β-hexachlorocyclohexane.

(1 min) −15 °C/min −280 °C (5 min). MS operating conditions were as follows: ionization method, electron impact; ion source temperature, 200 °C; detector gain, 1.50 kV; selected ion mode (p,p'-DDT, m/z 234.95; p,p'-DDE, m/z 246.00; HCB, m/z 283.80; β-HCH, m/z 180.95). Detection limits were as follows: p,p'-DDT, 0.005 ng/ml; p,p'-DDE, 0.002 ng/ml; HCB, 0.002 ng/ml; β-HCH, 0.006 ng/ml. Within-batch coefficients of variation ranged between approximately 15% and 20% based on 15 replicated measurements of plasma samples at a mean concentration (ng/ml) range of 0.120 (HCB) to 5.263 (p,p'-DDT). Cases and matched controls were assayed in the same batch by laboratory personnel who did not know the case–control status.

Serum total cholesterol and triglycerides were measured by enzymatic methods in each study area. Data for serum total cholesterol and triglycerides were available for 420 (97%) and 248 (57%) subjects, respectively. The precision and accuracy of cholesterol measurement in all laboratories was found to be satisfactory according to the Osaka Medical Center for Health Science and Promotion, a member of the Cholesterol Reference Method Laboratory Network (CRMLN) (Nakamura et al., 2003).

2.7. *Statistical analysis*

Linear regression analysis of log-transformed organochlorine values was performed to adjust for serum cholesterol concentration. We then used these adjusted values in our principal analysis of the 139 pairs for whom serum cholesterol measurements were available. In supplementary analyses, we used lipid-adjusted values calculated by the formula described by Philips et al. (Phillips et al., 1989) and crude values adjusted for serum cholesterol concentration in the conditional logistic regression model.

Comparison of baseline characteristics and plasma crude organochlorine levels between cases and controls was evaluated by the Mantel–Haenszel test using matched-pair strata. Using a conditional logistic regression model, we calculated odds rations (ORs) and 95% confidence intervals (CIs) of breast cancer for plasma organochlorine levels divided into quartiles based on the distribution among controls. The following variables were adjusted for as potential confounders: age at menarche (continuous), menopausal status at baseline

Table 3 – Odds ratios (ORs) and 95% confidence intervals (CIs) of breast cancer according to plasma organochlorine level

	Quartile category[a]				p for trend
	1	2	3	4	
p,p'-DDT (ng/ml), median[b]	0.50	0.89	1.36	2.24	
No. of cases/no. of controls	40/69	29/69	25/69	45/69	
OR (95% CI)	1.00	0.69 (0.36, 1.30)	0.61 (0.32, 1.19)	1.19 (0.63, 2.24)	0.64
Multivariate OR (95% CI)[c]	1.00	0.65 (0.32, 1.32)	0.56 (0.26, 1.22)	0.99 (0.47, 2.08)	0.97
p,p'-DDE (ng/ml), median[b]	2.50	4.83	7.58	14.41	
No. of cases/no. of controls	25/69	32/69	36/69	46/69	
OR (95% CI)	1.00	1.34 (0.69, 2.61)	1.56 (0.83, 2.93)	2.08 (1.09, 3.98)	0.02
Multivariate OR (95% CI)[c]	1.00	1.01 (0.47, 2.19)	1.24 (0.60, 2.53)	1.48 (0.70, 3.13)	0.25
HCB (ng/ml), median[b]	0.081	0.22	0.41	0.72	
No. of cases/no. of controls	43/69	25/69	35/69	36/69	
OR (95% CI)	1.00	0.57 (0.31, 1.06)	0.79 (0.44, 1.44)	0.82 (0.42, 1.60)	0.69
Multivariate OR (95% CI)[c]	1.00	0.46 (0.22, 0.95)	0.80 (0.40, 1.58)	0.82 (0.38, 1.76)	0.80
β-HCH (ng/ml), median[b]		Not detected	0.69	1.38	
No. of cases/no. of controls		70/138	32/69	37/70	
OR (95% CI)		1.00	0.91 (0.55, 1.52)	1.06 (0.62, 1.82)	0.91
Multivariate OR (95% CI)[c]		1.00	0.84 (0.45, 1.57)	0.74 (0.39, 1.39)	0.34

[a] Cholesterol-adjusted levels.
[b] Median crude plasma levels among the control group for p,p'-DDT, dichlorodiphenyltrichloroethane; p,p'-DDE, dichlorodiphenyldichloroethylene; HCB, hexachlorobenzene; and β-HCH, β-hexachlorocyclohexane.
[c] Adjusted for age at menarche (continuous), menopausal status at baseline (premenopausal women, age at menopause for postmenopausal women [≤47, 48–50, 51–53, ≥54]), number of births (0, 1, 2, 3, 4, 5+), age at first birth (≤21, 22–25, 26–29, ≥30, nulliparous), height (continuous), BMI (continuous), and alcohol consumption (non-drinkers, occasional drinkers, –50 (g/week), 50–100 (g/week), 100+ (g/week) among regular drinkers (ethanol)). Adjusted ORs were calculated based on a total of 370 subjects with complete information on covariates.

(premenopausal women, age at menopause for postmenopausal women [≤47, 48–50, 51–53, ≥54]), number of births (0, 1, 2, 3, 4, 5+), age at first birth (≤21, 22–25, 26–29, ≥30, nulliparous), height (continuous), body mass index (BMI) (continuous), and alcohol consumption (non-drinkers, occasional drinkers, –50 (g/week), 50–100 (g/week), 100+ (g/week) among regular drinkers (ethanol)). The linear trend of OR was tested in the conditional logistic regression model using the exposure categories as ordinal variables. All p values reported are two-sided, and significance level was set at $p<0.05$. All statistical analyses were performed with SAS software version 9.1 (SAS Institute, Inc., Cary, NC).

3. Results

Case subjects and controls had significantly different distributions for number of births (Table 1). Other characteristics, such as age at menarche, age at first birth, BMI, alcohol drinking, and dietary factors did not substantially differ between the groups.

p,p′-DDT and p,p′-DDE were detected in plasma in all subjects, and HCB and β-HCH in 91% and 73% of subjects, respectively. We found no statistically significant difference in plasma organochlorine levels between cases and controls (Tables 2 and 3). Further, no significant differences were seen on comparison of median concentrations of either estrogen receptor-positive (ER+) ($n=45$) or -negative (ER–) ($n=28$) cases and their respective controls according to analyses by the Mantel–Haenszel test using matched-pair strata. Median concentrations of plasma p,p′-DDE were 7.58 ng/ml for ER+ breast cancer cases and 6.22 ng/ml for their controls, and 6.60 ng/ml for ER- cases and 5.77 ng/ml for their controls.

Adjusted ORs for p,p′-DDT, HCB, and β-HCH were less than 1, and no statistical significance was observed. For p,p′-DDE, OR for the highest versus lowest quartile of plasma level was 2.08 (95% CI 1.09–3.98; p for trend=0.02) in the conditional model adjusted for matching factors only, but was attenuated after further adjustment for potential confounders to 1.48 (95% CI 0.70–3.13; p for trend=0.25). No substantial change was seen after further adjustment for other potential confounders such

Table 4 – Odds ratios (ORs) and 95% confidence intervals (CIs) of breast cancer according to plasma organochlorine level by baseline menopausal status[a]

	Quartile category[a]				P for trend
	1	2	3	4	
Premenopausal women					
p,p′-DDT (ng/ml)[b]					
No. of cases/no. of controls	12/21	12/37	11/25	20/27	
Multivariate OR (95% CI)[c]	1.00	0.48 (0.14, 1.58)	0.69 (0.19, 2.52)	2.45 (0.70, 8.63)	0.11
p,p′-DDE (ng/ml)[b]					
No. of cases/no. of controls	15/36	11/30	13/21	16/23	
Multivariate OR (95% CI)[c]	1.00	0.63 (0.19, 2.04)	1.78 (0.50, 6.32)	2.30 (0.73, 7.23)	0.08
HCB (ng/ml)[b]					
No. of cases/No. of controls	18/29	9/26	15/24	13/31	
Multivariate OR (95% CI)[c]	1.00	0.69 (0.25, 1.94)	0.91 (0.31, 2.63)	0.48 (0.14, 1.63)	0.34
β-HCH (ng/ml)[b]					
No. of cases/No. of controls		29/58	13/25	13/27	
Multivariate OR (95% CI)[c]		1.00	0.78 (0.29, 2.08)	0.68 (0.24, 1.94)	0.43
Postmenopausal women					
p,p′-DDT (ng/ml)[b]					
No. of cases/no. of controls	26/43	15/30	13/42	25/41	
Multivariate OR (95% CI)[d]	1.00	0.80 (0.29, 2.22)	0.39 (0.13, 1.21)	0.53 (0.18, 1.61)	0.17
p,p′-DDE (ng/ml)[b]					
No. of cases/no. of controls	10/32	17/35	23/44	29/45	
Multivariate OR (95% CI)[d]	1.00	1.56 (0.48, 5.06)	0.93 (0.32, 2.69)	1.07 (0.31, 3.63)	0.77
HCB (ng/ml)[b]					
No. of cases/no. of controls	23/38	15/39	18/42	23/37	
Multivariate OR (95% CI)[d]	1.00	0.35 (0.11, 1.05)	0.85 (0.32, 2.27)	1.09 (0.33, 3.54)	0.86
β-HCH (ng/ml)[b]					
No. of cases/no. of controls		38/73	18/41	23/43	
Multivariate OR (95% CI)[d]		1.00	0.61 (0.23, 1.63)	0.51 (0.19, 1.38)	0.19

[a] Cholesterol-adjusted levels.

[b] p,p′-DDT, dichlorodiphenyltrichloroethane; p,p′-DDE, dichlorodiphenyldichloroethylene; HCB, hexachlorobenzene; and β-HCH, β-hexachlorocyclohexane.

[c] Adjusted for age at menarche (continuous), number of births (0, 1, 2, 3, 4, 5+), age at first birth (≤21, 22–25, 26-29, ≥30, nulliparous), height (continuous), BMI (continuous), and alcohol consumption (non-drinkers, occasional drinkers, –50 (g/week), 50–100 (g/week), 100+ (g/week) among regular drinkers (ethanol)).

[d] Adjusted for age at menarche (continuous), age at menopause (≤47, 48–50, 51-53, ≥54), number of births (0, 1, 2, 3, 4, 5+), age at first birth (≤21, 22–25, 26–29, ≥30, nulliparous), height (continuous), BMI (continuous), alcohol consumption (non-drinkers, occasional drinkers, –50 (g/week), 50–100 (g/week), 100+ (g/week) among regular drinkers (ethanol)).

as family history of breast cancer, use of exogenous female hormones, leisure-time physical activity, and dietary intake of meat, fish, vegetables, fruits, energy and isoflavones. Moreover, they did not substantially change after exclusion of cases diagnosed within the first 3 years of follow-up. When we excluded subjects who provided a non-fasting blood sample, i.e. within 6 hours after a meal, no remarkable change was observed for *p,p'*-DDT, HCB, and β-HCH; however, the adjusted OR for the highest versus lowest quartile of plasma *p,p'*-DDE level was 2.71 (95% CI 0.92–7.96; p for trend = 0.06).

A stratified analysis according to baseline menopausal status showed no statistically significant association between plasma organochlorine level and the risk of breast cancer in any stratum (Table 4). In terms of *p,p'*-DDT and *p,p'*-DDE, however, positive associations were observed in premenopausal but not in postmenopausal women, although they were not statistically significant.

We further examined the association of the risk of breast cancer with the *p,p'*-DDT, *p,p'*-DDE, HCB, and β-HCH values adjusted for total serum lipids using the method of Phillips et al. (Phillips et al., 1989). Analyses were conducted on the 81 pairs for whom serum lipid measurements were available. We found no statistically positive association between these values and the risk of breast cancer (data not shown). In addition, no statistically positive association was observed by analyses using crude values adjusted for serum cholesterol concentration in the conditional logistic regression model (data not shown).

4. Discussion

Our data do not support the hypothesis that plasma levels of *p,p'*-DDT, *p,p'*-DDE, HCB, and β-HCH are associated with an increased risk of breast cancer among Japanese women overall. These findings are in general agreement with those of the majority of studies published from Western countries to date (Krieger et al., 1994; Lopez Carrillo et al., 1997; Hoyer et al., 1998; Dorgan et al., 1999; Helzlsouer et al., 1999; Aronson et al., 2000; Ward et al., 2000; Wolff et al., 2000; Laden et al., 2001a,b; Gammon et al., 2002; Lopez Cervantes et al., 2004; Raaschou Nielsen et al., 2005; Gatto et al., 2007). Our study, to our knowledge the first prospective study from an Asian country, suggests that, as in Western populations, exposure to organochlorines is unlikely to be an important cause of breast cancer in Asian populations.

To our knowledge, 11 prospective studies of the association between DDE level and the risk of breast cancer have been published (Wolff et al., 1993; Krieger et al., 1994; Hoyer et al., 1998; Dorgan et al., 1999; Helzlsouer et al., 1999; Hoyer et al., 2000; Ward et al., 2000; Wolff et al., 2000; Laden et al., 2001b; Raaschou Nielsen et al., 2005; Cohn et al., 2007). None of these studies found a significant positive association, however, apart from one early study by Wolff et al., which reported a four-fold elevation in breast cancer risk among women with the highest compared to the lowest serum level of DDE (Wolff et al., 1993). Six studies have investigated the association between DDT level and risk, with three reporting no association (Hoyer et al., 1998; Ward et al., 2000; Raaschou Nielsen et al., 2005), two reporting a significant positive (Hoyer et al., 2000; Cohn et al., 2007) and one reporting an inverse association (Dorgan et al., 1999). Further, none of the five studies which examined the association between β-HCH level and risk of breast cancer found a significant positive association (Hoyer et al., 1998; Dorgan et al., 1999; Hoyer et al., 2000; Ward et al., 2000; Raaschou Nielsen et al., 2005). Two studies reported an association with HCB level, a significant inverse association in one (Raaschou Nielsen et al., 2005) and a significant positive association in the other (Dorgan et al., 1999).

The range of DDE levels varied among studies, possibly reflecting the year of blood sample collection. For example, according to a combined analysis of five US studies, median concentrations in a control group were 11.13 ng/ml in samples collected in 1974 versus 2.63 ng/ml in samples collected in 1995-97 (Laden et al., 2001a). Levels in our study among Japanese women who provided samples in 1990–95 were comparable those in samples obtained in 1989–90 for the Nurses' Health Study in US, with median control concentrations of 6.08 ng/ml and 5.70 ng/ml, respectively (Laden et al., 2001b). These levels were measured more than 10 years after the use of DDT was banned. Although the long half-lives of the compounds and their resistance to metabolism means that current levels are used as a surrogate of past exposure, the major source of exposure to DDT has shifted from the more estrogenic *o,p'*-DDT found in technical DDT to the far less estrogenic *p,p'*-DDE occurring via the diet (Kelce et al., 1995; Soto et al., 1995). This shift is one possible explanation for the lack of association.

In this regard, one prospective study assessed serum samples collected from 1964 to 1971, when DDT was still heavily used in the US (Krieger et al., 1994). The mean concentration of serum DDE in the control group (43.1 ng/ml) was higher than those in the combined analysis of five US studies (Laden et al., 2001a), but again found no significant positive association between serum DDE levels and breast cancer risk overall. This finding in turn suggests that there is no association between DDE levels and breast cancer risk regardless of the source of exposure to DDT.

Given that the action of estrogen on breast cell proliferation appears to be mediated by estrogen receptors and that organochlorines are hypothesized to act as hormone mimics, any associations between organochlorines and breast cancer risk might differ by hormone receptor-defined subtype. An early study showed that the mean concentration of DDE in breast adipose tissue was significantly higher in women with ER+ breast cancer than in control women, but not in those with ER– breast cancer (Dewailly et al., 1994). Although this result suggests the possibility that DDE may influence breast cancer risk through estrogen, mediated by its receptor, it is not consistent with the majority of previous studies (Helzlsouer et al., 1999; Hoyer et al., 2001; Laden et al., 2001b; Raaschou Nielsen et al., 2005; Gatto et al., 2007). Among prospective studies, one study reported an inverse association between DDE in adipose tissue and the risk of ER- breast cancer (Raaschou Nielsen et al., 2005), while others showed no association between plasma DDE level and the risk of ER+ breast cancer (Laden et al., 2001b) or no difference in risk by hormone receptor-defined subtype (Helzlsouer et al., 1999; Hoyer et al., 2001). Moreover, a recent population-based case–

control study in the US also showed no difference in risk by hormone receptor-defined subtype (Gatto et al., 2007). In the present study, no significant difference in median concentrations was found between either ER+ or ER− breast cancer and their respective controls. Any interpretation of these results should be made with caution, however, because hormone receptor status was available for only 73 cases (53%).

Findings to date on the association of organochlorines and breast cancer risk stratified by menopausal status have been inconsistent. In the present study, *p,p'*-DDT and *p,p'*-DDE appeared to increase the risk among premenopausal but not among postmenopausal women. Similar findings were reported in a hospital-based case–control study of Canadian women: an elevated OR was observed in premenopausal women with the highest levels of breast adipose tissue DDE (OR=1.52, 95%CI 0.70–3.33) but no association was found in postmenopausal women (OR=1.05, 95%CI 0.50–2.19) (Aronson et al., 2000). Further, a hospital-based case–control study in Colombia found a higher OR for serum DDE level in premenopausal (highest tertile category compared with the lowest: OR=2.46, 95%CI 0.96–6.30) than in postmenopausal women (OR=1.85, 95%CI 0.85–4.05) (Olaya Contreras et al., 1998).

In contrast, Romieu et al's. population-based case–control study in Mexico City reported a significant association between serum DDE level and the risk of breast cancer in postmenopausal women (highest quartile category compared with the lowest: OR=5.26, 95%CI 0.80–34.30, *p* for trend=0.03) but no significant association in premenopausal women (OR=2.41, 95%CI 0.37–15.81, p for trend=0.16) (Romieu et al., 2000). Moreover, other studies found no remarkable difference between subgroups by menopausal status (Lopez Carrillo et al., 1997; Gammon et al., 2002). One reason for these inconsistent results is the relatively small numbers of cases in most of the subgroup analyses, i.e. less than 250 cases.

Our study has several methodological advantages. First, since blood samples were collected before cancer diagnosis in our nested case–control study within a prospective cohort, any potential bias due to the presence of cancer is likely obviated. Second, cases and controls were selected from the same cohort, thereby avoiding the selection bias inherent to case–control studies.

Several limitations of this study warrant discussion. First, because organochlorine levels in blood are influenced by circulating lipid levels, we adjusted organochlorine levels for serum cholesterol concentration in our primary analyses. Moreover, we matched case subjects and controls for fasting status at the time blood was drawn, limiting the potential effect of short-term variation in blood lipid levels. In contrast, a number of previous studies used an alternative lipid-adjustment method suggested by Phillips et al. (Phillips et al., 1989). In the present study, however, we used this method in our supplementary analyses because serum triglyceride measurement was available for only 57% of subjects. Second, in spite of a reasonably large cohort population (24,226 women) and long follow-up period (average 10.6 year), the number of breast cancer cases was relatively small, reflecting the low incidence rate in Japan (age-standardized rate per 100,000 world population, 32.7 in 2002) (Ferlay et al., 2004). The interpretability of our results might therefore be limited, particularly in stratified analyses. Third, although our cohort subjects were selected from the general population, subjects were restricted to the 24,226 women (43%) respondents to the baseline questionnaire who provided blood samples. Thus, any extrapolation of the results to the general population should be done cautiously, particularly in view of our previous report showing the difficulty of extrapolating relative risk estimates for a sub-cohort to an entire cohort (Iwasaki et al., 2006). This difficulty might in fact be inherent to prospective studies in general.

In conclusion, this study suggests that there is no overall association between organochlorines at the levels measured in plasma and the risk of breast cancer among Japanese women. It is unlikely that exposure to these compounds can explain the increasing rates of breast cancer in Japan.

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EPIDEMIOLOGY

Dietary isoflavone intake and breast cancer risk in case–control studies in Japanese, Japanese Brazilians, and non-Japanese Brazilians

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Abstract Although epidemiologic studies have shown an inverse association between isoflavones and breast cancer risk, little evidence for a dose–response relation is available. We conducted hospital-based case–control studies of patients aged 20–74 years with primary, incident, histologically confirmed invasive breast cancer, and matched controls from medical checkup examinees in Nagano, Japan and from cancer-free patients in São Paulo, Brazil. A total of 850 pairs (390 Japanese, 81 Japanese Brazilians and 379 non-Japanese Brazilians) completed validated food frequency questionnaires. The odds ratio of breast cancer according to isoflavone intake was estimated using a conditional logistic regression model. We found a statistically significant inverse association between isoflavone intake and the risk of breast cancer for Japanese Brazilians and non-Japanese Brazilians. For Japanese, a non-significant inverse association was limited to postmenopausal women. In the three populations combined, breast cancer risk linearly decreased from 'no' to 'moderate' isoflavone intake and thereafter leveled off. Compared to non-consumers, adjusted odds ratios (95% confidence interval) for consumers in increasing quintile intake categories (median intake in each category: 8.7, 23.1, 33.8, 45.7, and 71.3 mg/day) were 0.69 (0.44–1.09), 0.54 (0.31–0.94), 0.45 (0.26–0.77), 0.34 (0.19–0.62), and 0.43 (0.24–0.76), respectively. Overall, we found an inverse association between dietary isoflavone intake and risk of breast cancer. Our finding suggests a risk-reducing rather than risk-enhancing effect of isoflavones on breast cancer within the range achievable from dietary intake alone. In addition, women may benefit

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from risk reduction if they consume at least moderate amounts of isoflavones.

Keywords Breast cancer · Dietary isoflavones · Case–control study · Immigrants

Abbreviations

CI	Confidence interval
ER	Estrogen receptor
FFQ	Food-frequency questionnaire
OR	Odds ratio
PR	Progesterone receptor

Introduction

Soy foods, which are rich in isoflavones, are habitually consumed by Asian populations in large amounts. Isoflavones, of which genistein and daidzein are major examples, are classified as phytoestrogens, which are plant-derived non-steroidal compounds with estrogen-like biological properties. A high intake of isoflavones has therefore been hypothesized to contribute to the lower incidence of breast cancer in Asia than Western countries [1]. This hypothesis is supported by not only in vitro studies at high genistein concentrations and the majority of animal studies [2, 3] but also epidemiological studies [4–10]. In particular, a recent meta-analysis showed a small decrease in risk of breast cancer with higher soy intake [11] while a more recent meta-analysis indicated that risk reduction was limited to Asian populations [12]. In apparent contradiction to potential protective effects, however, genistein exhibits estrogenic properties at low concentrations, which could theoretically enhance breast cancer risk [2, 3], and some animal studies have in fact reported that genistein stimulates tumor development and growth [13, 14].

Although research remains insufficient for any comprehensive determination of whether isoflavones are protective or harmful for breast cancer, interest in soy foods and isoflavones is nevertheless increasing. This increase may reflect an expectation of potential benefits in a wide variety of medical conditions, including cancer of the endometrium and prostate as well as breast, cardiovascular diseases, osteoporosis, and menopausal symptoms. In fact, consumption of soy foods in the United States has increased over the past ten years, against fairly constant intake in Japan over the past four decades [15]. Moreover, phytoestrogen supplements are commercially marketed for use by postmenopausal women as natural and safe alternatives to hormone replacement therapy. A dose–response pattern, in particular the effect of relatively high-dose isoflavones on breast cancer risk, is thus now of concern. Nevertheless, little evidence of any dose–response relationship is available—indeed, we do not know the answer to 'how much isoflavones is needed?' This is partly because few studies have estimated isoflavone intake using a validated food-frequency questionnaire (FFQ) [4–6, 16, 17], and also because most studies in Western countries have involved only a small variation in isoflavone intake [6, 7, 16–20].

Here, to evaluate the dose–response relationship between isoflavone intake and the risk of breast cancer, ranging from zero to the relatively high levels achievable from dietary intake only, we conducted hospital-based case–control studies in Nagano, Japan and São Paulo, Brazil, areas with a low and middle incidence of breast cancer, respectively (age-standardized rate per 100,000 world population, 32.7 and 46.0 in 2002, respectively) [21], using validated FFQs with relatively high validity in three populations: Japanese living in Japan, Japanese Brazilians living in São Paulo, and non-Japanese Brazilians living in São Paulo. The mortality of breast cancer among these three populations has increased over the last 20 years, with that in Japanese Brazilians intermediate between that in Japanese and Brazilians [22]. In addition, because amounts and variations in isoflavone intake are expected to be high and large for Japanese, intermediate and relatively large for Japanese Brazilians, and low and small for non-Japanese Brazilians, respectively, these populations serve as suitable venues for studies of the effect of dose–response relations.

Materials and methods

Study subjects

These multicenter, hospital-based case–control studies of breast cancer were designed to determine lifestyle factors and genetic susceptibility to the risk of breast cancer and to compare potential risk factors among Japanese living in Nagano, Japan, and Japanese Brazilians and non-Japanese Brazilians living in São Paulo, Brazil. Eligible cases were a consecutive series of female patients aged 20–74 years with newly diagnosed and histologically confirmed invasive breast cancer. Cases were recruited between 2001 and 2005 at four hospitals in Nagano, and between 2001 and 2006 at eight hospitals in São Paulo. A total of 405 cases (98%) participated in Nagano, and 83 Japanese Brazilians (91%) and 389 non-Japanese Brazilians (99%) in São Paulo. In the study in Nagano, eligible controls were selected from medical checkup examinees in two of the four hospitals and confirmed not to have cancer. One control was matched for each case by age (within 3 years) and residential area during the study period. Among potential controls, one examinee refused to participate and two refused to provide blood samples. Consequently, we

obtained written informed consent from 405 matched pairs. In the study in São Paulo, eligible controls were preferentially selected from cancer-free patients who visited the same hospital as the index cases. One control was matched for each case by age (within 5 years) and ethnicity during the study period. Among potential controls, 22 patients refused to participate (participation rate = 96%). Consequently, we obtained written informed consent from 472 matched pairs (83 for Japanese Brazilians and 389 for non-Japanese Brazilians). The study protocol was approved by CONEP (Comissão Nacional de Ética em Pesquisa), Brasília, Brazil and by the institutional review board of the National Cancer Center, Tokyo, Japan.

Data collection

Participants in Nagano were asked to complete a self-administered questionnaire, while in-person interviews were conducted by trained interviewers using a structured questionnaire in São Paulo. The two questionnaires contained closely similar questions concerning demographic characteristics, medical history, family history of cancer, menstrual and reproductive history, anthropometric factors, physical activity, and smoking habits. For dietary habits, we used a semi-quantitative FFQ (136 items for the Japanese version and 118 items for the Brazilian version) which was developed and validated in each population [23, 24]. Information on estrogen receptor (ER) and progesterone receptor (PR) status was obtained from medial records. Hormone receptor status was determined by either enzyme-linked immunoassay or immunohistochemical assay. Hormone receptor positivity values were determined either as specified by the laboratory that performed the assay, or in accordance with the laboratory's written interpretation thereof, or both.

Dietary assessment

In the FFQ, participants were questioned on how often they consumed the individual food items (frequency of consumption), as well as relative sizes compared to standard portions. Response choices for frequency were never or less than once/month, 1–3 times/month, 1–2 times/week, 3–4 times/week, 5–6 times/week, once/day, 2–3 times/day, 4–6 times/day, and 7 times/day or more, and relative sizes to a standard portion were small (50% smaller than standard), medium (same as standard), and large (50% larger). For the Japanese version, white rice intake was determined in terms of the relative size of the rice bowl used and the frequency of intake, with the nine choices of less than 1–10 bowls per day. Frequency for miso soup intake was given in the six choices of almost never, 1–3 times/month, 1–2 times/week, 3–4 times/week, 5–6 times/week, or daily, while amount was given in nine categories ranging from less than 1–10 bowls per day, without reference to the relative size of the bowl used. Daily food intake was calculated by multiplying frequency by standard portion and relative size for each food item in the FFQ. Daily intakes of genistein and daidzein were calculated using a food composition table of isoflavones developed previously [25, 26]. Isoflavone intake was defined for this study as the sum of genistein and daidzein intake. Other nutrients were calculated using the Japanese Standard Tables of Food Composition, 5th ed. for the Japanese version [27] and the United States Department of Agriculture (USDA) food composition tables for the Brazilian version [28]. For some Japanese-specific foods in the Brazilian version, the Japanese Standard Tables of Food Composition, 5th ed. was used.

The validity of isoflavone intake estimated from the Japanese version of the FFQ was evaluated in a subsample of the Japan Public Health Center-based Prospective Study, which includes Nagano as one of the study areas. The estimated intake according to the FFQ was compared to that in four consecutive 7-day dietary records, one conducted in each the four seasons. Spearman's correlation coefficients between energy-adjusted genistein and daidzein intake estimated from the FFQ and from dietary records were 0.59 for genistein and 0.60 for daidzein [24]. For the Brazilian version, the validity of isoflavone intake estimated from the FFQ was evaluated in a subsample of the control group in this case–control study by comparing the estimated intake according to the FFQ to that in two consecutive 4-day dietary records, one each in two seasons. Spearman's correlation coefficients between energy-adjusted genistein and daidzein intake estimated from the FFQ and from dietary records were 0.76 for genistein and 0.76 for daidzein (unpublished data).

Statistical analysis

We excluded subjects who reported extremely low or high total energy intake (<500 or ≥ 4000 Kcal), leaving 390 pairs of Japanese, 81 pairs of Japanese Brazilians and 379 pairs of non-Japanese Brazilians for use in the present analyses. Comparison of baseline characteristics between cases and controls was evaluated by the Mantel-Haenszel test using matched-pair strata in each population. Dietary intake of isoflavones was adjusted for total energy intake by the residual method and divided into median or tertile categories based on control distribution for Japanese and Japanese Brazilians, respectively. Because of the small proportion of consumers, non-Japanese Brazilians were categorized into non-consumers and consumers of isoflavones. Using a conditional logistic regression model, we calculated odds ratios (ORs) and 95% confidence intervals

(CIs) of breast cancer for isoflavone intake. An unconditional logistic regression model was used for stratified analyses according to menopausal status. Associations between isoflavone intake and hormone receptor-defined breast cancer were assessed by an unconditional polytomous logistic regression model. Linear trends for ORs were tested in the logistic regression model using the exposure categories as ordinal variables. The following variables, which were mainly selected based on comparison of baseline characteristics between cases and controls, were adjusted for as potential confounders: menopausal status, number of births, family history of breast cancer, smoking status, moderate physical activity in the past 5 years, and vitamin supplement use. We did not include a history of benign breast disease as a covariate since we regarded it as an intermediate variable in the causal pathway between isoflavone intake and breast cancer. All p values reported are two-sided, and significance level was set at $P < 0.05$. All statistical analyses were performed with SAS software version 9.1 (SAS Institute, Inc., Cary, NC).

Results

Characteristics of cases and controls and isoflavone intake (Table 1)

For Japanese, the proportion of premenopausal women, current smokers, and vitamin supplement users was higher in cases than in controls, and cases tended to have a family history of breast cancer and history of benign breast disease. Cases were less likely than controls to breast-feed, be physically active, and eat vegetables. For Japanese Brazilians, cases were less likely than controls to give birth and be physically active and more likely to eat vegetables and fruits. For non-Japanese Brazilians, the proportion of premenopausal women and current smokers was higher in cases than controls while the proportion of physically active women and vitamin supplement users was lower. Isoflavone intake substantially varied among populations, with mean intakes (mg/day) in control subjects of 46.1 for Japanese, 24.9 for Japanese Brazilians, and 4.4 for non-Japanese Brazilians. Because genistein and daidzein intakes were highly correlated, with a Spearman's correlation coefficient for the three populations of 0.99, only isoflavone intake was used for the following analyses.

ORs in the three populations (Table 2)

We found a statistically significant inverse association between isoflavone intake and the risk of breast cancer for Japanese Brazilians and non-Japanese Brazilians but not for Japanese. Adjusted OR for the highest versus lowest tertile of isoflavone intake was 0.25 (95% CI 0.09–0.68; P for trend <0.01) for Japanese Brazilians. For non-Japanese Brazilians, adjusted OR for consumers versus non-consumers of isoflavones was 0.56 (95% CI 0.35–0.90). No substantial change was seen after further adjustment for other potential confounders, such as age at menarche, age at menopause, age at first birth, history of breast feeding, body mass index, alcohol drinking, or vegetable and fruit intake.

A stratified analysis according to menopausal status revealed that an inverse association was limited to postmenopausal women in Japan although it was not statistically significant. Adjusted OR for the highest versus lowest tertile of isoflavone intake was 0.62 (95% CI 0.38–1.01; P for trend = 0.06) for postmenopausal women, but 1.35 (95% CI 0.72–2.54; P for trend = 0.41) for premenopausal women. The inverse association was stronger in premenopausal than postmenopausal women for Japanese Brazilians but no remarkable difference between the two strata was seen for non-Japanese Brazilians.

ORs of hormone receptor-defined breast cancer (Table 3)

Information on the combined ER and PR status of the breast tumor was available for 387 (99%) Japanese, 61 (75%) Japanese Brazilians, and 264 (70%) non-Japanese Brazilians cases. The following subtypes were used for modeling in an unconditional polytomous logistic regression model: positive for both receptors (ER+/PR+), ER-positive and PR-negative (ER+/PR−), and negative for both receptors (ER−/PR−) for Japanese, and ER+/PR+, ER+/PR−, ER−/PR−, and unknown for Japanese Brazilians and non-Japanese Brazilians. Overall, we found no remarkable difference in risk by hormone receptor-defined subtype.

Dose–response pattern (Table 4; Fig. 1)

To evaluate dose–response relations using a wide range of isoflavone intake, we combined individual study data from three populations and categorized the subjects into six groups, namely non-consumers and quintiles among isoflavone consumers based on the combined control distribution. Compared to non-consumers, adjusted ORs (95% CI) for consumers in increasing quintile categories (median intake in each category: 8.7, 23.1, 33.8, 45.7, and 71.3 mg/day) based on a conditional logistic regression model were 0.69 (0.44–1.09), 0.54 (0.31–0.94), 0.45 (0.26–0.77), 0.34 (0.19–0.62), and 0.43 (0.24–0.76), respectively. A stratified analysis according to menopausal status based on an unconditional logistic regression model revealed that this inverse association was more prominent in postmenopausal

Table 1 Characteristics of case and matched control subjects

	Japanese living in Nagano, Japan			Japanese Brazilians living in São Paulo, Brazil			Non-Japanese Brazilians living in São Paulo, Brazil		
	Case ($n = 390$)	Control ($n = 390$)	P^a	Case ($n = 81$)	Control ($n = 81$)	P^a	Case ($n = 379$)	Control ($n = 379$)	P^a
Age (years), mean	53.8	54.0	–	56.6	56.5	–	52.4	52.5	–
Premenopausal women, %	46	35	<0.01	31	30	0.80	42	38	0.04
Age at menopause (years), mean[b]	49.0	49.4	0.15	49.9	50.6	0.73	49.1	48.4	0.13
Age at menarche (years), mean[b]	13.4	13.2	0.42	12.9	12.9	0.20	13.2	13.1	0.96
Nulliparous women, %	13	14	0.66	23	16	0.24	11	10	0.91
Number of births (≥4 births), %	2	3	0.16	7	20	0.02	29	35	0.10
Age at first birth (years), mean[b, c]	26.9	26.4	0.42	28.6	27.5	0.25	23.2	22.5	0.24
Breast feeding (yes), %[c]	91	96	0.03	92	91	0.56	88	91	0.67
Oral contraceptives user, %	3	3	1.00	29	36	0.30	63	65	0.62
Family history of breast cancer, %	11	6	0.02	15	12	0.65	6	6	0.88
History of benign breast disease, %	12	7	0.03	12	6	0.17	7	7	1.00
Height (cm), mean[b]	155.3	155.5	0.50	154.0	153.9	0.91	158.2	158.4	0.96
Body mass index (kg/m^2), mean[b]	22.7	23.0	0.07	24.3	24.5	0.43	26.6	26.1	0.11
Smoking (current smoker), %	8	5	<0.01	11	2	0.07	17	11	0.04
Alcohol drinking (regular drinker), %	26	29	0.25	2	6	0.26	6	6	0.65
Moderate physical activity past 5 years (yes), %	32	40	0.02	19	32	0.03	9	14	0.03
Vitamin supplement user, %	18	12	0.03	19	26	0.27	3	9	<0.01
Total energy intake (kcal/day), mean[b]	1881.6	1949.3	0.27	1662.0	1587.7	0.44	1847.0	1752.8	0.09
Fish and shellfish intake (g/day), mean[b]	87.6	94.4	0.11	27.4	30.5	0.56	13.7	16.6	0.24
Meat or red meat intake (g/day), mean[b, d]	58.1	57.6	0.36	54.3	53.3	0.44	72.1	64.2	0.14
Vegetable intake (g/day), mean[b]	257.6	310.5	<0.01	146.7	93.0	<0.01	77.7	86.4	0.96
Fruit intake (g/day), mean[b]	288.6	287.7	0.69	364.0	311.0	0.02	260.2	250.9	0.35
Isoflavone intake (mg/day), mean[b]	43.5	46.1	<0.01	16.5	24.9	0.15	1.1	4.4	0.01
Genistein intake (mg/day), mean[b]	27.0	28.6	<0.01	10.2	15.8	0.15	0.73	3.1	0.01
Daidzein intake (mg/day), mean[b]	16.5	17.5	<0.01	6.3	9.1	0.15	0.33	1.4	0.01

[a] P for Mantel-Haenszel test with matched-pair strata

[b] Adjusted for age

[c] Among parous women

[d] Meat intake for Japanease and red meat intake for Japanese Brazilians and non-Japanese Brazilians

than premenopausal women. To clarify the effect of high isoflavone intake in detail, subjects were further categorized into 11 groups, namely non-consumers and deciles of isoflavone consumers. We found a linear decrease in breast cancer risk from zero to moderate intake (20–30 mg/day) and a leveling-off thereafter based on a conditional logistic regression model (Fig. 1). No increasing trend was found for relatively high intake.

Discussion

In these case–control studies of Japanese, Japanese Brazilians, and non-Japanese Brazilians, overall, we found an inverse association between dietary isoflavone intake and the risk of breast cancer. Our finding is in general agreement with those of a recent meta-analysis [11] and in five of the ten previous studies examining the association between isoflavone intake as estimated by FFQ and breast cancer risk [4–8]. It is noteworthy that, although several experimental studies have suggested adverse effects from soy constituents [2, 3, 13, 14], no epidemiological study estimating isoflavone intake by FFQ has reported an increased risk of breast cancer. Our study also suggests a risk-reducing rather than risk-enhancing effect of isoflavones on breast cancer within the range achievable from dietary intake alone. It remains unclear, however, whether isoflavone exposure other than dietary intake is associated with the risk of breast cancer.

We found a linear decrease in breast cancer risk from zero to moderate intake (20–30 mg/day) and thereafter a leveling-off. This dose–responses pattern might imply the

Table 2 Odds ratios (ORs) and 95% confidence intervals (CIs) of breast cancer according to dietary isoflavone intakes

	Median isoflavone intake[a]	All subjects						Premenopausal women				Postmenopausal women			
		No.		OR[b]	95% CI	OR[c]	95% CI	No.		OR[d]	95% CI	No.		OR[d]	95% CI
		Case	Control					Case	Control			Case	Control		
Japanese living in Nagano, Japan															
Tertile 1	22.1	152	129	1.00		1.00		80	67	1.00		72	62	1.00	
Tertile 2	39.6	118	131	0.75	(0.53–1.07)	0.86	(0.59–1.27)	52	44	0.99	(0.58–1.71)	66	87	0.79	(0.48–1.29)
Tertile 3	69.1	120	130	0.75	(0.52–1.10)	0.83	(0.54–1.28)	46	26	1.35	(0.72–2.54)	74	104	0.62	(0.38–1.01)
P for trend				0.12		0.39				0.41				0.06	
Japanese Brazilians living in São Paulo, Brazil															
Tertile 1	4.7	41	26	1.00		1.00									
Tertile 2	13.4	25	28	0.51	(0.23–1.15)	0.48	(0.20–1.16)								
Tertile 3	42.8	15	27	**0.35**	**(0.15–0.80)**	**0.25**	**(0.09–0.68)**								
P for trend				**0.01**		**<0.01**									
Median 1	8.0	48	40	1.00		1.00		16	10	1.00		32	30	1.00	
Median 2	35.0	33	41	0.68	(0.37–1.26)	0.52	(0.26–1.06)	9	14	**0.17**	**(0.03–0.84)**	24	27	0.84	(0.37–1.92)
Non-Japanese Brazilians living in São Paulo, Brazil															
Non-consumers	0	343	318	1.00		1.00		147	124	1.00		196	194	1.00	
Consumers	15.0	36	61	**0.54**	**(0.34–0.84)**	**0.56**	**(0.35–0.90)**	14	21	0.54	(0.26–1.13)	22	40	0.58	(0.33–1.03)

[a] Crude intake (mg/day)

[b] Crude OR

[c] Conditional model adjusting for menopausal status (premenopausal women, postmenopausal women), number of births (0, 1, 2, 3, 4, 5+), family history of breast cancer (yes, no), smoking status (never, past, current smokers), moderate physical activity in the past 5 years (no, less than 3 days/month, 1–4 days/week, more than 5 days/week), and vitamin supplement use (yes, no)

[d] Unconditional model adjusting for matching factors (age and area for Japanese; age and hospital for Japanese Brazilians; age and ethnicity for non-Japanese Brazilians), number of births (0, 1, 2, 3, 4, 5+), family history of breast cancer (yes, no), smoking status (never, past, current smokers), moderate physical activity in the past 5 years (no, less than 3 days/month, 1–4 days/week, more than 5 days/week), and vitamin supplement use (yes, no)

Bold characters indicates statistically significant values

Table 3 Odds ratios (ORs) and 95% confidence intervals (CIs) of hormone receptor-defined breast cancer according to dietary isoflavone intakes

	No. of controls	ER+/PR+			ER+/PR−			ER−/PR−			Unknown		
		No. of cases	OR[a]	95% CI	No. of cases	OR[a]	95% CI	No. of cases	OR[a]	95% CI	No. of cases	OR[a]	95% CI
Japanese living in Nagano, Japan, all subjects													
Tertile 1	129	82	1.00		23	1.00		38	1.00				
Tertile 2	131	70	0.98	(0.64–1.51)	24	1.10	(0.58–2.08)	21	0.58	(0.32–1.07)			
Tertile 3	130	67	0.97	(0.62–1.51)	22	0.71	(0.36–1.43)	28	0.71	(0.40–1.28)			
P for trend			0.89			0.35			0.23				
Japanese living in Nagano, Japan, premenopausal women													
Tertile 1	67	46	1.00		8	1.00		18	1.00				
Tertile 2	44	40	1.35	(0.74–2.46)	4	0.80	(0.22–2.89)	6	0.47	(0.17–1.32)			
Tertile 3	26	27	1.51	(0.74–3.07)	7	1.64	(0.48–5.58)	10	0.94	(0.34–2.56)			
P for trend			0.22			0.52			0.65				
Japanese living in Nagano, Japan, postmenopausal women													
Tertile 1	62	36	1.00		15	1.00		20	1.00				
Tertile 2	87	30	0.68	(0.37–1.25)	20	1.25	(0.57–2.73)	15	0.65	(0.30–1.44)			
Tertile 3	104	40	0.68	(0.38–1.22)	15	0.53	(0.22–1.26)	18	0.57	(0.27–1.22)			
P for trend			0.21			0.14			0.15				
Japanese Brazilians living in São Paulo, Brazil, all subjects													
Median 1	40	24	1.00		7	1.00		7	1.00		9	1.00	
Median 2	41	16	0.63	(0.27–1.45)	2	0.22	(0.04–1.36)	4	0.34	(0.08–1.49)	11	1.24	(0.38–4.03)
Non-Japanese Brazilians living in São Paulo, Brazil, all subjects													
Non-consumers	318	97	1.00		41	1.00		76	1.00		108	1.00	
Consumers	61	8	0.46	(0.21–1.004)	9	1.10	(0.50–2.41)	10	0.67	(0.33–1.40)	7	**0.35**	**(0.16–0.80)**

[a] Unconditional model adjusting for matching factors (age and area for Japanese; age and hospital for Japanese Brazilians; age and ethnicity for non-Japanese Brazilians), menopausal status (premenopausal women, postmenopausal women), number of births (0, 1, 2, 3, 4, 5+), family history of breast cancer (yes, no), smoking status (never, past, current smokers), moderate physical activity in past 5 years (no, less than 3 days/month, 1–4 days/week, more than 5 days/week), and vitamin supplement use (yes, no)

Bold characters indicates statistically significant values

Table 4 Odds ratios (ORs) and 95% confidence intervals (CIs) of breast cancer according to dietary isoflavone intake based on combined individual study data from three populations

	Non-consumers and quintile category among consumers						*P* for trend
	0	1	2	3	4	5	
Median isoflavone intake (mg/day)[a]	0	8.7	23.1	33.8	45.7	71.3	
Japanese living in Nagano, Japan							
No. of cases/No. of controls	0/0	49/31	93/90	89/85	72/96	87/88	
Japanese Brazilians living in São Paulo, Brazil							
No. of cases/No. of controls	9/5	46/41	16/12	3/8	1/6	6/9	
Non-Japanese Brazilians living in São Paulo, Brazil							
No. of cases/No. of controls	343/318	27/33	5/3	2/13	2/3	0/9	
All subjects in three populations							
No. of cases/No. of controls	352/323	122/105	114/105	94/106	75/105	93/106	
OR	1.00	0.69	**0.54**	**0.45**	**0.34**	**0.43**	**<0.01**
(95% CI)[b]		(0.44–1.09)	**(0.31–0.94)**	**(0.26–0.77)**	**(0.19–0.62)**	**(0.24–0.76)**	
Premenopausal women in three populations							
No. of cases/No. of controls	150/127	48/37	58/52	49/37	23/30	36/23	
OR	1.00	0.68	0.44	0.54	**0.27**	0.62	0.27
(95% CI)[c]		(0.33–1.39)	(0.19–1.01)	(0.24–1.24)	**(0.10–0.69)**	(0.25–1.54)	
Postmenopausal women in three populations							
No. of cases/No. of controls	202/196	74/68	56/53	45/69	52/75	57/83	
OR	1.00	0.70	0.52	**0.31**	**0.34**	**0.33**	**<0.01**
(95% CI)[c]		(0.40–1.24)	(0.26–1.04)	**(0.15–0.64)**	**(0.17–0.71)**	**(0.16–0.66)**	

[a] Energy adjusted by residual method

[b] Conditional model adjusting for menopausal status (premenopausal women, postmenopausal women), number of births (0, 1, 2, 3, 4, 5+), family history of breast cancer (yes, no), smoking status (never, past, current smokers), moderate physical activity in the past 5 years (no, less than 3 days/month, 1–4 days/week, more than 5 days/week), and vitamin supplement use (yes, no)

[c] Unconditional model adjusting for age (continuous), study population (Japanese living in Nagano, Japan; Japanese Brazilians living in São Paulo, Brazil; non-Japanese Brazilians living in São Paulo, Brazil), number of births (0, 1, 2, 3, 4, 5+), family history of breast cancer (yes, no), smoking status (never, past, current smokers), moderate physical activity in the past 5 years (no, less than 3 days/month, 1–4 days/week, more than 5 days/week), and vitamin supplement use (yes, no)

Bold characters indicates statistically significant values

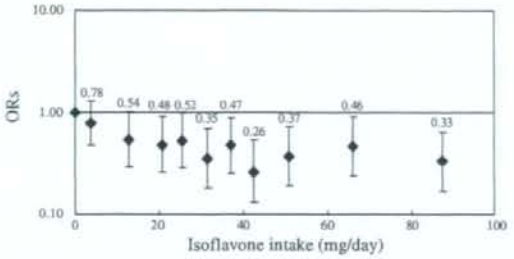

Fig. 1 Odds ratios (ORs) and 95% confidence intervals of breast cancer according to dietary isoflavone intake based on combined individual data from three populations. Subjects were categorized into 11 groups: non-consumers and deciles of isoflavone consumers based on the control distribution. ORs were estimated using matching pairs with adjustment for menopausal status (premenopausal women, postmenopausal women), number of births (0, 1, 2, 3, 4, 5+), family history of breast cancer (yes, no), smoking status (never, past, current smokers), moderate physical activity in the past 5 years (no, less than 3 days/month, 1–4 days/week, more than 5 days/week), and vitamin supplement use (yes, no)

presence of a ceiling effect and suggests that women may benefit from risk reduction if they consume at least a moderate amount of isoflavones. Alternatively, it might merely reflect differences in measurement errors due to the use of different FFQs, selection bias, and residual confounding among the three populations, notwithstanding that it clearly reflected the results of separate analyses. Specifically, consumers had lower risk than non-consumers in non-Japanese Brazilians, whose average intake of isoflavone was 4.4 mg/day among the control group; the risk of breast cancer decreased with increasing intake of isoflavone in Japanese Brazilians, whose average intake of isoflavone was 24.9 mg/day among the control group; while higher intake of isoflavone was not associated with further risk reduction in Japanese, whose average intake of isoflavone was 46.1 mg/day among the control group. Confirmation of this pattern would require further prospective cohort studies using blood or urine samples as an exposure assessment, because these could minimize the measurement errors and selection bias mentioned above.

Our stratified analysis by menopausal status using data from the three populations combined showed that an inverse association was more prominent among postmenopausal than premenopausal women. In addition, our separate analyses showed somewhat different patterns in the three populations: the inverse association was limited to postmenopausal women in Japanese; it was stronger in premenopausal than postmenopausal women in Japanese Brazilians; and no remarkable difference was found in non-Japanese Brazilians. These findings are inconsistent with a recent meta-analysis showing an inverse association regardless of menopausal status [11]. Moreover, findings to date on the association of isoflavone intake and the risk of breast cancer stratified by menopausal status have been inconsistent, with one prospective cohort study in Japan [4] and one case–control study in the United States [8] reporting that an inverse association was limited to postmenopausal women; one case–control study in Japan [5] showing it was limited to premenopausal women; and one prospective cohort study in the United States [16] and three case–control studies [6, 17, 18] finding no difference between the two strata.

Several mechanisms by which isoflavones may reduce the risk of breast cancer have been proposed [2, 3]. The most prominent and thoroughly investigated mechanisms are mediated via estrogen receptors, arising due to the similar chemical structure of isoflavones to the human estrogen hormone and their binding affinity to estrogen receptors [3, 29]. Given that the action of estrogen on breast cell proliferation appears to be mediated by estrogen receptors, therefore, any association between isoflavone intake and breast cancer risk might differ by hormone receptor-defined subtype. The present study did not support this hypothesis, however, showing no apparent difference in risk by subtype. Moreover, results for the few studies to date have been inconsistent [7, 16, 18, 19]. Although our findings might merely be explained by a lack of statistical power, they suggest that the anti-cancer effects of isoflavones might be evoked not only by mechanisms mediated by estrogen receptors but also by other mechanisms, such as the modulation of endogenous hormones via inhibition of the key enzyme involved in estrogen biosynthesis and metabolism; the arrest of cell cycle progression; induction of apoptosis; inhibition of tyrosine kinase activity, topoisomerase II activity, and angiogenesis; and antioxidant activity [2, 3].

Our study has several methodological advantages over previous studies of isoflavones and the risk of breast cancer. First, isoflavone intake differed considerably among the three populations, with median levels (interquartile rage) in the control group (mg/day) of 40.6 (25.9–61.2) among Japanese, 13.4 (8.1–35.0) among Japanese Brazilians, and 0 (0–0) among non-Japanese Brazilians. This range allowed the detailed evaluation of dose–response relations, ranging from zero to a relatively high level achievable from dietary intake only, and is unique to the present study. Second, the overall consistency of findings in the three populations allowed for the greater generalizability of results as compared to those from a single population.

Several limitations of this study warrant mention. First, dietary intake of isoflavone was assessed after the diagnosis of breast cancer and is therefore sensitive to recall bias. Second, although the substantially high participation rates among both eligible cases and controls minimized potential biases related to control selection, the use of controls from

medical checkup examinees and cancer-free patients, whose dietary habits may differ from the general population due to health consciousness or disease, might have lead to selection bias. Third, stratified analyses were performed based on a relatively small number of cases. The interpretability of our results might therefore be limited.

Allowing for these methodological issues, we found an inverse association between dietary isoflavone intake and the risk of breast cancer in case–control studies of Japanese, Japanese Brazilians, and non-Japanese Brazilians. Our findings suggest a risk-reducing rather than risk-enhancing effect of isoflavones on breast cancer within the range achievable from dietary intake alone. In addition, women may benefit from risk reduction if they consume at least moderate amounts of isoflavones.

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ORIGINAL PAPER

Serum organochlorines and breast cancer risk in Japanese women: a case–control study

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Abstract
Objective Most epidemiological studies of the association between breast cancer risk and exposure to organochlorine pesticides or polychlorinated biphenyls (PCBs), which are suspected endocrine disrupters and potential risk factors for human breast cancer, have been conducted in western countries, and the majority of results have been null and the rest inconsistent. Here, we examined these associations in Japanese women in the largest study in Asian women to date.

Methods The study was a matched case–control study of breast cancer with 403 eligible matched pairs from May 2001 to September 2005 at four hospitals in Nagano Prefecture, Japan.
Measurements Serum samples were measured for PCBs and nine pesticide-related organochlorines, including dichlorodiphenyltrichloroethane (DDT). Odds ratios of breast cancer or its hormone-receptor-defined subtypes according to serum organochlorines were calculated.
Results No increase in the risk of breast cancer was seen among women with higher serum concentrations of any organochlorine: *o,p'*-DDT, *p,p'*-DDT, *p,p'*-dichlorodiphenyldichloroethylene, hexachlorobenzene, *β*-hexachlorocyclohexane, *trans*-nonachlor, *cis*-nonachlor, oxychlordane, mirex, or PCBs. Rather, higher serum levels of *cis*-nonachlor, mirex, or total PCBs were associated with a decreased risk of breast cancer
Conclusions Overall, these results suggest that breast cancer risk in Japan, a low-incidence country, is similar to that in western countries in terms of organochlorine exposure.

Keywords Hormone receptor · PCBs · DDT · DDE · Mirex

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Introduction

Breast cancer is the most frequent malignant disease among women in many western countries, and also in Japan [1]. Epidemiological evidence for the occurrence of breast cancer has suggested an association with several estrogen-dependent factors, namely early menarche, lower parity, late age at first childbirth, postmenopausal hormone use, taller height, and obesity [2–5]. This hormonal

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dependency of breast cancer plays an important role in its development.

Because established risk factors for breast cancer account for only about half of its incidence [6–8]; however, epidemiological studies have also examined certain organochlorine pesticides, such as dichlorodiphenyltrichloroethane (DDT) and polychlorinated biphenyls (PCBs), which have shown estrogenicity or anti-estrogenicity in experimental studies [9], as potential risk factors. Results have been mainly null, with several inconsistent exceptions [6, 10]. Some of these agents persist in the environment and are bio-accumulative. Release of organochlorine pollutants into the environment during part of the last century has resulted in exposure in humans, mainly via food intake, particularly fish [11]. Although the human body burden of traditional organochlorine compounds such as DDTs and PCBs has decreased in recent decades following bans on their use, they remain detectable in biologic samples [12].

Most studies of associations of breast cancer risk with particular organochlorine compounds to date have been conducted among Caucasians in western countries. Such associations among relatively high-risk Caucasians in these countries may not always be consistent with those among lower-risk Asian women in Asian countries such as Japan, however [13]. Indeed, the possibility that differences in background, such as in lifestyle, internal hormonal milieu, body burden of organochlorines, and hormone-receptor subtypes, might influence the associations have now made race, ethnicity, and area specificity issues in this field. In recent years, three studies have assessed the association between breast cancer and organochlorines in African-American women [14–16], one of which also included Asian subjects (50 pairs) [15], but all reported null results; and apart from one small study in India (25 pairs), no study has yet been conducted in an Asian country, including Japan [17].

Here, we conducted a matched case–control study to investigate the association between breast cancer risk and serum organochlorines in Japanese women. This is the first large-scale study to examine the association between breast cancer and DDTs, PCBs, and other organochlorine pesticides in an Asian country and an Asian population.

Subjects and methods

Study subjects

This multicenter, hospital-based case–control study was conducted from May 2001 to September 2005 at four hospitals in Nagano Prefecture, Japan. Cases were a consecutive series of women aged 20–74 years with newly arising, histologically confirmed invasive breast cancer admitted to the four hospitals during the survey period. Out of the 412 eligible patients, 405 (98%) agreed to participate. Healthy controls were selected from among medical checkup examinees in two of the hospitals, who were confirmed as not having cancer, with one control matched for each case by age (within 3 years) and residential area during the study period. Among potential control subjects, one declined to participate. Consequently, we obtained written informed consent from 405 matched pairs. Because two control subjects refused to provide blood samples, the analysis was finally restricted to 403 matched pairs. The study protocol was approved by the institutional review board of the National Cancer Center (Tokyo, Japan).

Data collection

Questionnaire survey

Participants were asked to complete a self-administered questionnaire which included questions on demographic characteristics, anthropometric factors, smoking habits, family history of cancer, physical activity, medical history, and menstrual and reproductive history. Dietary habits were investigated using a 136-item semi-quantitative food-frequency questionnaire (FFQ) which was developed and validated in Japanese population [18]. The FFQ enquired about the frequency of consumption of individual food items, with response choices of never or less than once/month, 1–3 times/month, 1–2 times/week, 3–4 times/week, 5–6 times/week, once/day, 2–3 times/day, 4–6 times/day, and 7 times/day or more; as well as relative sizes compared to standard portions, expressed as small (50% smaller than standard), medium (same as standard), and large (50% larger). These data were then used to calculate average rates of consumption for each food group (g/day) and nutrients (mg/day).

Clinical data

Estrogen receptor (ER) and progesterone receptor (PR) status in breast cancer tissue of breast cancer patients were obtained from medical records. Hormone receptor status was determined using enzyme-linked immunoassay or immunohistochemical assay. Hormone receptor positivity values were determined either as specified by the laboratory that performed the assay, or in accordance with the laboratory's written interpretation thereof, or both.

Laboratory analysis

Blood specimens were collected from all cancer patients prior to surgery. Aliquots of serum samples were shipped to a commercial laboratory, Shimadzu Techno Research

Inc. (Kyoto, Japan), for analysis of *o,p'*-DDT, *p,p'*-DDT, *p,p'*-dichlorodiphenyldichloroethylene (*p,p'*-DDE), hexachlorobenzene (HCB), β-hexachlorocyclohexane (β-HCH), *trans*-nonachlor, *cis*-nonachlor, oxychlordane, mirex, and more than 41 PCB congeners. An approximately 1.5-g aliquot of each serum sample was gravimetrically measured and then spiked with respective ^{13}C-labelled internal standards, except for some PCB congeners. Compounds were fractionated and purified using two-stage liquid–liquid extraction with dichloromethane, ethanol and saturated ammonium sulfate aqueous solution, and florisil column chromatography.

Organochlorine pesticides and PCBs were detected and measured using a high-resolution mass spectrometer (Autospec Ultima, Micromass, Manchester, UK) with selected ion monitoring connected to a gas chromatograph (HP6890, Hewlett Packard) equipped with a capillary column (HT8-PCB fused silica capillary column 60 m × 0.25-mm id for PCBs; DB-17HT fused silica capillary column 30 m × 0.32 mm id, 0.15 μm for the organochlorine pesticides) based on isotope-dilution mass spectrometry.

Lower limits of detection (LODs), determined from a signal-to-noise ratio of 3, were 1.0 pg/g wet for organochlorine pesticides and 0.6 pg/g wet for PCB congeners. Measurement values below the LOD were assigned a value equal to the LOD. Total PCBs was calculated as the sum concentration of all PCB congeners measured. Cases and matched controls were assayed in the same batch by laboratory analysts who did not know the case–control status. Some compounds were detected in the method blank but were disregarded because of their sufficiently low contribution to measured values in serum concentrations or the sum of PCBs. Intraclass correlation coefficients derived from duplicate measures of five serum samples at usual concentration ranges were >0.99, except for that for HCB, which was 0.87.

To standardize serum concentrations of lipophilic organochlorines, measurement values were divided by serum total lipid concentration (TL), which was estimated using serum concentrations of total cholesterol (TC) and triglyceride (TG), and the equation TL [g/l] = 2.27 × TC [g/l] + TG [g/l] + 0.623, as proposed by Phillips et al. [19], and then divided by serum density (g/l). TC and TG values were enzymatically measured at Kyoto Biken Laboratories, Inc. (Kyoto, Japan).

Statistical analysis

Statistical analyses were performed using SAS 9.1 (SAS Institute Inc., Cary, NC, USA). All statistical tests and 95% confidence intervals (CIs) were two-sided and considered significant at the 0.05 level. Serum lipid-adjusted organochlorine concentrations were categorized into quartile groups based on the distribution of controls. Missing variables were handled by complete case analysis. Twelve pairs which included women with a total energy intake of less than 500 kcal or 4,000 kcal or more were excluded from adjustment of odds ratios (ORs) for fish or vegetable consumption.

ORs and 95% CIs of breast cancer according to quartile of serum organochlorine concentration were calculated by conditional logistic regression analysis using the PHREG procedure with the STRATA statement in SAS. Stratified analyses by menopausal status or age were performed using unconditional logistic regression analysis. ORs were adjusted for total lipid concentration in serum, body-mass index, menopausal status and age at menopause, smoking status, fish consumption, vegetable consumption, family history of breast cancer in a first-degree relative, age at first childbirth, parity, age at menarche, history of breast cancer screening, and history of breast feeding. These adjusted variables were established risk factors for breast cancer or were correlated with breast cancer risk and serum organochlorines in this study. Owing to the significant inverse association between risk and total lipid concentration in serum, logistic regression models were additionally adjusted for serum lipids. Because lactation results in the excretion of organochlorines, a longer total duration of lactation may be associated with increased excretion of organochlorines [20]. In addition, the unconditional logistic regression models used for stratified analyses were also adjusted for the matching factors of age and area of residence.

To evaluate differences in the effect of organochlorines on breast cancer by hormone-receptor subtype, each breast cancer case was categorized by the combined classification of ER and PR status as follows: ER−PR−, ER−PR+, ER+PR−, or ER+PR+. ER−PR+ cases were excluded owing to their small number ($n = 12$). Polytomous logistic regression was performed based on a generalized logit model using the LOGIST procedure in SAS. The difference in the beta coefficient for ordinal variables used in the trend test by hormone receptor status was simultaneously tested using the Wald test in polytomous logistic regression models.

Along with the logistic regression analyses described above, we also performed linear trend tests to examine the monotonicity of the dose–response relationship based on median value in each quartile category of serum organochlorine concentration as an ordinal variable.

Results

Case and control characteristics are summarized in Table 1. The cases included a higher percentage of premenopausal women than the matched controls. Among