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Plasma Isoflavone Level and Subsequent Risk of Breast Cancer Among Japanese Women: A Nested Case-Control Study From the Japan Public Health Center-Based Prospective Study Group

Motoki Iwasaki, Manami Inoue, Tetsuya Otani, Shizuka Sasazuki, Norie Kurahashi, Tsutomu Miura, Seiichiro Yamamoto, and Shoichiro Tsugane

From the Epidemiology and Prevention Division, Research Center for Cancer Prevention and Screening, National Cancer Center; Department of Sport and Exercise Nutrition, School of Physical Education, Sendai University; and the Cancer Information Services and Surveillance Division, Center for Cancer Control and Information Services, National Cancer Center, Tokyo, Japan.

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Corresponding author: Motoki Iwasaki, MD, PhD, Epidemiology and Prevention Division, Research Center for Cancer Prevention and Screening, National Cancer Center, 5-1-1 Tsukiji, Chuo-ku, Tokyo, 104-0045, Japan; e-mail: moiwasak@gan2.res.ncc.go.jp.

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ABSTRACT

Purpose
Because they have large variations in consumption, Asian countries are suitable settings for studies of the effect of relatively high-dose isoflavone intake on breast cancer risk. Nevertheless, no prospective study from Asia has assessed blood or urine levels as biomarkers of isoflavone intake.

Patients and Methods
A total of 24,226 women ages 40 to 69 years in the Japan Public Health Center–based prospective study who responded to the baseline questionnaire and provided blood in 1990 to 1995 were observed to December 2002. During a mean 10.6 years of follow-up, 144 patients newly diagnosed with breast cancer were identified. Two matched controls for each patient were selected from the cohort. Isoflavone levels were assessed by plasma level and food frequency questionnaire, and the odds ratio of breast cancer according to isoflavone level was estimated using a conditional logistic regression model.

Results
We found a statistically significant inverse association between plasma genistein and risk of breast cancer, but no association for plasma daidzein. Adjusted odds ratios for the highest versus lowest quartile of plasma level were 0.34 for genistein (95% CI, 0.16 to 0.74; *P* for trend, .02) and 0.71 for daidzein (95% CI, 0.35 to 1.44; *P* for trend, .54). Median plasma genistein values in the control group were 31.9 ng/mL for the lowest and 353.9 ng/mL for the highest quartile groups. Regarding dietary intake of isoflavones, nonsignificant inverse associations were observed for both genistein and daidzein.

Conclusion
This nested case-control study found an inverse association between plasma genistein and the risk of breast cancer in Japan.

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INTRODUCTION

Soy foods, a traditional staple dish in Asian countries, are a primary source of isoflavones, such as genistein and daidzein, which are classified as phytoestrogens. Because breast cancer risk is substantially lower in Asian than Western countries,[1] the contribution of a high isoflavone intake to low breast cancer risk has been hypothesized.[2] This hypothesis has been supported by in vitro studies at high genistein concentrations and in the majority of animal studies, which together have demonstrated various anticancer effects of isoflavones acting via both estrogen-dependent and -independent mechanisms.[3,4] Estrogen-dependent mechanisms arise through the mediation of estrogen receptor α and β, owing to the similar chemical structure of isoflavones to the human estrogen hormone and their binding affinity to estrogen receptors.[4,5] For this reason, they have been hypothesized to behave like selective estrogen receptor modulators. In contradiction to potential protective effects, however, genistein exhibits estrogenic properties at low concentrations, which could theoretically enhance breast cancer risk.[3,4] In fact, some animal studies have reported that genistein stimulates tumor development and growth.[6,7] Although a recent meta-analysis found that soy intake was associated with a

small reduction in breast cancer risk, the authors concluded that in view of these risk-enhancing effects, recommendations for high-dose isoflavone supplementation to prevent breast cancer or its recurrence were premature.[8] Phytoestrogen supplements, however, are commercially marketed for use by postmenopausal women as natural and safe alternatives to hormone replacement therapy. The effect of relatively high-dose isoflavone on breast cancer risk is now of concern.

Because they have large variations in consumption among individuals, Asian countries serve as suitable venues for studies of the effect of relatively high-dose isoflavone intake on breast cancer risk. Despite this advantage, only a few epidemiological studies on soy or isoflavone intake and breast cancer risk from Asia have been reported.[9] In particular, no prospective study on isoflavone levels in blood or urine samples has been reported, notwithstanding that, because they are partly determined by individual differences in absorption and metabolism, blood or urine levels might better reflect interperson differences than dietary assessment. The three nested case-control studies which have investigated this association in Western populations have been inconsistent, with one reporting an inverse association with plasma genistein in the Netherlands,[10] the second showing no association with urinary genistein in the Netherlands,[11] and the third finding a positive association with urine and serum phytoestrogens in the United Kingdom.[12] This inconsistency might be in part explained by the apparently small variation in isoflavone levels in Western countries. For example, studies in the Netherlands, which has a high incidence of breast cancer (age-standardized rate per 100,000 world population, 86.7 in 2002),[13] reported a median genistein intake of 0.14 mg/d in women ages 49 to 70 years,[14] and a median plasma genistein level of 4.89 ng/mL in the control group of a nested-case control study.[10] In contrast, a study in Japan, where the incidence of breast cancer is low (age-standardized rate per 100,000 world population, 32.7 in 2002),[13] reported a median genistein intake of 22.3 mg/d and median serum level of 90.2 ng/mL.[15] This substantial variation in isoflavone levels suggests that the Japanese population represents an ideal setting for determining whether an association exists at relatively high levels achievable from dietary intake only.

Herein, to clarify the effect of relatively high-dose isoflavone exposure on breast cancer risk, we conducted a nested case-control study within a large-scale population-based prospective study in Japan.

PATIENTS AND METHODS

Study Population

The Japan Public Health Center–based prospective study, which began in 1990 for cohort I and in 1993 for cohort II, included 140,420 subjects (68,722 men and 71,698 women) living in the municipalities supervised by 11 public health centers (PHC). Details of the study design have been described elsewhere.[16] The study protocol was approved by the institutional review board of the National Cancer Center, Tokyo, Japan.

The study population comprised registered Japanese inhabitants living in each PHC area, ages 40 to 59 years in cohort I and 40 to 69 years in cohort II. In this analysis, one PHC area was excluded since data on cancer incidence were not available. We thus defined a population-based cohort of 67,426 women (27,389 in cohort I and 40,037 in cohort II) after the exclusion of ineligible subjects (n = 95).

Questionnaire Survey

A baseline survey was conducted from 1990 to 1994. A total of 55,891 women (83%) returned the questionnaire, which contained questions concerning demographic characteristics, medical history, menstrual and reproductive history, anthropometric factors, physical activity, smoking and drinking habits, and diet.

Blood Collection

Subjects voluntarily provided 10 mL of blood during health check-ups from 1990 to 1995. Blood samples were divided into plasma and buffy layers and stored at −80°C until analysis. Among respondents to the baseline questionnaire, a total of 24,996 women (45%) donated blood.

Follow-Up

All registered subjects were observed 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), 1,289 subjects (5.2%) moved out of the study area and 5 (0.02%) were lost to follow-up within the study at-risk period.

Selection of Patients and Controls

Incidence data on breast cancer were collected for the Japan Public Health Center cancer registry through two data sources—major local hospitals and population-based cancer registries. Death certificates were used to supplement information on cancer incidence. Site of origin and histologic type were coded by members of our study group (Appendix A1, online only) using the International Classification of Diseases for Oncology, third edition, code C500-509. Up to the end of the study period, 144 new breast cancer cases (97 in cohort I and 47 in cohort II) were identified among the 24,226 women (9,689 in cohort I and 14,537 in cohort II) who had returned the baseline questionnaire, reported no history of breast cancer or ovarian cystoma, and provided blood samples. Diagnosis was microscopically verified in 98% of patients, and based on death certificates only in 0.7%. The mortality/incidence ratio was 0.14.

For each patient, two controls were selected using incidence density sampling from subjects who were not diagnosed with breast cancer during the follow-up period when the patient was diagnosed. Control selection was done without reference to incidence of other cancer sites. Controls were matched with each patient for 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 baseline menopausal status.

Assessment of Dietary Intake

Dietary intakes of genistein and daidzein were assessed by a food frequency questionnaire of 44 items for cohort I and 52 for cohort II. Isoflavone intake was defined for this study as the sum of genistein and daidzein intake. We documented the questionnaire assessment of isoflavone intake to be reasonably valid (details in Appendix A1).[15,17]

Laboratory Assay

Plasma levels of isoflavone were analyzed using high-performance liquid chromatography with a coulometric array detector in accordance with the modified methods of Gamache and Acworth.[18] Concentrations of genistein and daidzein were determined by linear regression of the peak height for each standard, and adjusted according to the recovery rate of the internal plasma standard. The regression coefficient of peak height and concentration calculated for isoflavones revealed a linearity range of 0 to 0.75 μg/mL, with correlation coefficient values higher than 0.938. Voltametric response for the standard solution displayed coefficients of variation of 8% for intra- and 11% for interday variation. Recovery rates of isoflavones in plasma samples ranged between approximately 73% and 98%. Detection limits were 2.2 ng/mL for genistein and 2.7 ng/mL for daidzein. Laboratory personnel were blinded to case-control status when performing the analyses.

Statistical Analysis

Comparison of baseline characteristics, as well as plasma levels and dietary intake of isoflavones, between cases and controls was evaluated by the Mantel-Haenszel test using matched-set strata. Spearman's correlation coefficients were calculated among plasma levels and dietary intakes of isoflavone

among control subjects. Using a conditional logistic regression model, we calculated odds ratios (ORs) and 95% CIs of breast cancer for plasma levels and dietary intake of isoflavone divided into quartiles based on control distribution. The ORs were adjusted for number of births and age at first birth as potential confounders. The adjusted ORs were calculated based on a total of 405 subjects with complete information for covariates. Linear trends for ORs were 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 $P < .05$. All statistical analyses were performed with SAS software, version 9.1 (SAS Institute Inc, Cary, NC).

RESULTS

Case subjects and controls had significantly different distribution for number of births (Table 1). Other characteristics, such as age at menarche, age at first birth, body mass index (BMI), alcohol consumption, or dietary intake did not substantially differ between the two groups.

Plasma genistein was significantly lower among cases than controls whereas plasma daidzein values were similar (Table 2). No significant differences between the groups were seen for dietary genistein, daidzein, or isoflavone intake. Median isoflavone intake in the control group was 34.8 mg/d (36.1 in cohort I and 29.9 mg/d in cohort II). Genistein and daidzein were highly correlated for both plasma level ($r = 0.72$) and dietary intake ($r = 0.99$). Correlation coefficients between plasma and dietary levels were relatively low for both genistein ($r = 0.23$) and daidzein ($r = 0.31$).

We found a statistically significant inverse association between plasma genistein and the risk of breast cancer (P for trend, .02), but no statistically significant association for plasma daidzein (P for trend, .54; Table 3). Adjusted ORs for the highest versus lowest quartile of plasma level were 0.34 for genistein (95% CI, 0.16 to 0.74; $P \leq .01$) and 0.71 for daidzein (95% CI, 0.35 to 1.44; $P = .34$). Moreover, the results did not change substantially after adjustment for dietary intake of isoflavone or other potential confounders such as age at menarche, menopausal status at baseline, age at menopause, height, BMI, and alcohol consumption. Further, exclusion of cases diagnosed before the first 3 years of follow-up did not substantially change the results, nor did the exclusion of subjects who used vitamin supplements or who provided a nonfasting blood sample (ie, within 6 hours after a meal). Regarding dietary intake, we observed inverse associations for both genistein and daidzein but neither was statistically significant (Table 3). In addition, adjusted ORs by isoflavone intake were closely similar to those by genistein intake (data not shown).

A stratified analysis according to baseline menopausal status showed no remarkable difference between two strata for either genistein and daidzein, regardless of whether the values were assessed by plasma or questionnaire, although the inverse association between plasma genistein and risk of breast cancer tended to be more stable in postmenopausal than premenopausal women (Table 4).

Table 1. Characteristics of Patients and Matched Control Subjects at Baseline

Characteristic	Patients (n = 144) No.	Patients (n = 144) %	Controls (n = 288) No.	Controls (n = 288) %	P*
Mean age, years	51.7		51.8		
Standard deviation	7.1		7.1		—
Family history of breast cancer	2	1.4	2	0.7	.48
Premenopausal women	59	42	118	42	—
Postmenopausal women					
Natural menopause	70	50	140	50	—
Surgical menopause	10	7.2	20	7.2	—
Mean age at menopause, years	50.0		49.8		.76
SE†	0.38		0.27		
Mean age at menarche, years	14.6		14.8		.33
SE†	0.15		0.10		
Mean No. of births	2.3		2.8		.01
SE†	0.12		0.09		
Mean age at first birth, years	25.7		25.0		.22
SE†	0.30		0.21		
Use of exogenous female hormones (current use)	4	3.0	2	0.8	.10
Mean height, cm	151.7		151.4		.70
SE†	0.46		0.33		
Mean body mass index, kg/m^2	23.4		23.5		.49
SE†	0.25		0.18		
Smoking (current smoker)	5	3.5	17	5.9	.23
Alcohol drinking (regular drinker)	18	13	26	9.1	.28
Leisure-time physical activity (≥ once per week)	30	21	57	20	.42
Vitamin supplement user	33	24	61	23	.65
Green tea intake (≥ five cups per day)	36	25	71	25	.42
Mean total energy intake, kcal/d	1,269.4		1,271.0		.41
SE‡	26.5		19.2		
Mean fish and shellfish intake, g/d	45.4		45.7		.75
SE‡	2.5		1.8		
Mean meat intake, g/d	30.5		28.5		.15
SE‡	1.7		1.2		
Mean vegetable intake, g/d	121.2		115.9		.20
SE‡	5.7		4.1		
Mean fruit intake, g/d	104.8		99.4		.79
SE‡	5.9		4.3		

*P for Mantel-Haenszel test with matched-set strata.
†Adjusted for age.
‡Adjusted for age and cohort.

DISCUSSION

In this study, we found a statistically significant inverse association between plasma genistein and the risk of breast cancer, but no association for plasma daidzein. This finding suggests that genistein may

Table 2. Plasma Levels and Dietary Intake of Isoflavone in Patients and Matched Controls

Parameter	Patients (n = 144) Median	Patients (n = 144) Interquartile Range	Controls (n = 288) Median	Controls (n = 288) Interquartile Range	P*
Plasma level					
Genistein, ng/mL	131.8	67.9-202.6	144.5	78.8-255.6	.046
Daidzein, ng/mL	16.7	7.0-34.0	17.9	5.5-40.8	.45
Dietary intake					
Genistein, mg/d	19.9	16.6-24.0	21.7	16.8-26.1	.37
Daidzein, mg/d	12.5	10.1-14.8	13.3	10.3-16.3	.36
Isoflavone, mg/d†	32.5	26.8-38.7	34.8	27.0-42.4	.36

*P for Mantel-Haenszel test with matched-set strata.
†Isoflavone intake = sum of genistein and daidzein intake.

Table 3. ORs and 95% CIs of Breast Cancer According to Plasma Level and Dietary Intake of Isoflavone

Parameter	Quartile 1	2	3	4	*P* for trend
Plasma level					
Median genistein, ng/mL	31.9	108.1	190.8	353.9	
No. of patients	41	37	45	21	
No. of controls	72	72	72	72	
OR	1.00	0.84	1.04	0.46	.07
95% CI	Reference	0.47 to 1.51	0.57 to 1.91	0.23 to 0.91	
Adjusted OR*	1.00	0.69	0.87	0.34	.02
95% CI	Reference	0.36 to 1.32	0.45 to 1.67	0.16 to 0.74	
Median daidzein, ng/mL	0	12.0	27.0	53.7	
No. of patients	30	45	44	25	
No. of controls	72	72	72	72	
OR	1.00	1.50	1.44	0.79	.59
95% CI	Reference	0.85 to 2.64	0.80 to 2.61	0.41 to 1.54	
Adjusted OR*	1.00	1.30	1.51	0.71	.54
95% CI	Reference	0.70 to 2.42	0.80 to 2.86	0.35 to 1.44	
Dietary intake					
Median genistein, mg/d	15.7	18.5	22.9	27.3	
No. of patients	42	36	37	29	
No. of controls	69	75	71	73	
OR	1.00	0.78	0.83	0.58	.15
95% CI	Reference	0.46 to 1.35	0.47 to 1.48	0.30 to 1.12	
Adjusted OR*	1.00	0.81	0.92	0.58	.21
95% CI	Reference	0.46 to 1.45	0.50 to 1.70	0.29 to 1.18	
Median daidzein, mg/d	9.4	11.4	14.1	17.1	
No. of patients	40	39	35	30	
No. of controls	70	74	72	72	
OR	1.00	0.91	0.82	0.65	.21
95% CI	Reference	0.52 to 1.58	0.46 to 1.47	0.33 to 1.27	
Adjusted OR*	1.00	0.96	0.94	0.67	.34
95% CI	Reference	0.54 to 1.74	0.50 to 1.74	0.33 to 1.39	

Abbreviation: OR, odds ratio.
*Adjusted for number of births (0, 1, 2, 3, 4, 5+) and age at first birth (−21, 22-25, 26-29, 30+, nulliparous). Adjusted ORs were calculated based on a total of 405 subjects with complete information of covariates.

play a more important role in the etiology of breast cancer than daidzein. Our findings are in general agreement with those of a recent nested case-control study in the Netherlands,[10] albeit that our inverse association occurred at substantially higher plasma concentrations. For example, median plasma genistein values in the control group of the Netherlands study were 3.75 ng/mL for premenopausal and 4.89 ng/mL for postmenopausal women.[10] In contrast, the median value in our control group was 144.5 ng/mL, and only 3.2% of control subjects was under 5 ng/mL. This apparently high level is not surprising considering that the median value of 353.9 ng/mL in our highest plasma genistein quartile group, which had a significantly lower risk of breast cancer than the lowest group, corresponded to a median dietary intake of 28.5 mg/d for genistein and 46.5 mg/d for isoflavone, as estimated by the validation study data. Although some in vivo and in vitro studies have shown risk-enhancing effects of genistein, our study suggests that relatively high-dose isoflavones exposure achievable from dietary intake alone is associated with a decreased rather than increased risk.

We observed an approximately 65% reduction in breast cancer risk in the highest plasma genistein quartile group but no decrease in the other quartiles, indicating that only the highest group benefited from risk reduction. The apparent lack of a dose-response relationship might imply the presence of a threshold level of effect. Interestingly, this idea contradicts findings in Western populations, in whom inverse associations are seen despite materially low levels of isoflavones. Given the differences in hormonal milieu between the two populations, the potential protective effect of isoflavones in breast cancer might act differently between Western and Asian populations: sex hormone levels are higher in Western than Asian women,[19] for example, as is the prevalence of obesity.[20,21] In this regard, a case-control study in Shanghai found that the inverse association between urinary isoflavone level and breast cancer risk was stronger among women in the high BMI, waist-hip ratio, and estradiol level groups and in the low sex hormone-binding globulin level group than in the respectively converse low and high groups.[22] Alternatively, the apparent lack of a dose-response relationship might merely reflect uncontrolled confounding by other dietary characteristics or risk-lowering behaviors.

The reason for a role for genistein but not daidzein in the etiology of breast cancer is unclear, but several possibilities can be speculated. Genistein possesses stronger binding affinity for estrogen receptor than daidzein.[5] Further, a pharmacokinetic study showed higher plasma levels and a 1.5-fold longer half-life for genistein than daidzein

Table 4. ORs and 95% CIs of Breast Cancer According to Plasma Level and Dietary Intake of Isoflavone By Baseline Menopausal Status

Parameter	Quartile 1	2	3	4	*P* for trend
Premenopausal women					
Plasma genistein, ng/mL					
No. of patients	24	14	19	2	
No. of controls	41	28	25	24	
Adjusted OR*	1.00	0.76	1.75	0.14	.20
95% CI	Reference	0.31 to 1.86	0.68 to 4.50	0.03 to 0.69	
Plasma daidzein, ng/mL					
No. of patients	17	21	15	6	
No. of controls	27	45	23	23	
Adjusted OR*	1.00	0.80	1.27	0.49	.48
95% CI	Reference	0.34 to 1.88	0.48 to 3.38	0.15 to 1.57	
Dietary genistein intake, mg/d					
No. of patients	21	16	14	8	
No. of controls	35	31	32	20	
Adjusted OR*	1.00	0.92	0.86	0.62	.43
95% CI	Reference	0.41 to 2.05	0.34 to 2.18	0.21 to 1.84	
Dietary daidzein intake, mg/d					
No. of patients	20	17	14	8	
No. of controls	36	30	32	20	
Adjusted OR*	1.00	1.07	0.93	0.67	.53
95% CI	Reference	0.46 to 2.51	0.37 to 2.34	0.22 to 2.03	
Postmenopausal women					
Plasma genistein, ng/mL					
No. of patients	17	23	25	15	
No. of controls	28	41	46	45	
Adjusted OR*	1.00	0.54	0.57	0.36	.10
95% CI	Reference	0.18 to 1.62	0.20 to 1.65	0.12 to 1.12	
Plasma daidzein, ng/mL					
No. of patients	13	23	27	17	
No. of controls	40	27	47	46	
Adjusted OR*	1.00	2.86	2.06	1.16	.95
95% CI	Reference	1.03 to 7.98	0.82 to 5.17	0.43 to 3.15	
Dietary genistein intake, mg/d					
No. of patients	20	20	22	18	
No. of controls	33	42	35	50	
Adjusted OR*	1.00	0.73	0.93	0.52	.31
95% CI	Reference	0.30 to 1.77	0.38 to 2.27	0.19 to 1.42	
Dietary daidzein intake, mg/d					
No. of patients	19	22	20	19	
No. of controls	33	42	36	49	
Adjusted OR*	1.00	0.89	0.93	0.64	.43
95% CI	Reference	0.38 to 2.10	0.38 to 2.29	0.23 to 1.72	

Abbreviation: OR, odds ratio.
*Adjusted for number of births (0, 1, 2, 3, 4, 5+) and age at first birth (−21, 22-25, 26-29, 30+, nulliparous).

after ingestion of baked soybean powder containing closely similar amounts of the two.[23] Moreover, the absence of an association for plasma daidzein might be attributable to misclassification arising from the metabolization of this compound. Daidzein can be metabolized by intestinal bacteria to equol and O-desmethylangolites; because approximately only 30% to 50% of individuals are capable of equol production, probably due to differences in gut microflora, daidzein-to-equol metabolizers may have lower plasma daidzein levels than nonmetabolizers.[24] Equol has been suggested to have greater biologic activity than daidzein,[24] and an inverse association between equol level and breast cancer risk has been reported.[25] Here, the lowest plasma daidzein quartile group might conversely have had a lower breast cancer risk than the higher groups due to its inclusion of equol metabolizers, and such misclassification, if present, would lead to a null result.

Our study has several methodological advantages over previous studies of isoflavones and the risk of breast cancer. First, the direct measurement of plasma isoflavone levels provides not only an index of intake but also of the absorption and metabolism of isoflavone, an understanding of which is important to elucidating the mechanisms by which isoflavones might influence breast cancer development. Indirect measurement by dietary intake of genistein is likely a major reason for the present smaller and nonsignificant risk reduction of breast cancer than by plasma genistein. Exposure assessment using

blood samples is therefore likely a more sophisticated means of detecting an association. Second, two case-control studies in Australia and China showed an inverse association between urinary isoflavones and breast cancer risk.[25,26] In view of the retrospective design of these studies, however, blood or urine levels of isoflavones in breast cancer cases might have been influenced by metabolic changes after the breast cancer was detected or by altered eating habits among case subjects. In our nested case-control study within a prospective cohort, in contrast, blood samples were collected before cancer diagnosis, obviating any potential bias due to the presence of cancer. Third, 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 mention. First, we measured plasma isoflavones only once for each individual. The consumption of soy foods is a personal dietary preference, and intake levels of most individuals are assumed to be relatively stable over time in Japan, as suggested by our validation study, which showed high reproducibility of repeated measurements of genistein intake by food frequency questionnaire (correlation coefficient = 0.72 for 1-year interval and 0.61 for 5-year interval).[15,17] By comparison, plasma isoflavone levels may reflect short-term rather than long-term intake: isoflavones have short half-lives in blood (eg, 6 to 8 hours),[23,27] and plasma levels are particularly affected by time elapsed since the last meal. To minimize the attenuation of risk estimates derived from random measurement errors, we matched fasting time between cases and controls. Second, despite a reasonably large cohort population (24,226 women) and long follow-up period (average, 10.6 years), 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).[13] 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 respondents (43%) to the baseline questionnaire who provided blood samples. Although health check-up examinees in our previous report had a different socioeconomic status than nonexaminees and a more favorable lifestyle profile,[28] no apparent difference in isoflavone intake and breast cancer risk factors was found between subjects in the subcohort for this study and the original cohort; median isoflavone intake, for example, was 32.5 and 32.1 mg/d, respectively, and the average number of births was 2.8 and 2.7, respectively.[29] Nevertheless, any extrapolation of the results to the general population should be done cautiously, particularly in view of a previous report showing the difficulty of extrapolating relative risk estimates for a subcohort to an entire cohort. This difficulty might in fact be inherent to prospective studies in general.[30]

Allowing for these methodological issues, we found an inverse association between plasma genistein and the risk of breast cancer in a nested case-control study in Japan. This finding suggests a risk-reducing rather than a risk-enhancing effect of isoflavones on breast cancer, even at relatively high concentrations within the range achievable from dietary intake alone.

AUTHORS' DISCLOSURES OF POTENTIAL CONFLICTS OF INTEREST

The author(s) indicated no potential conflicts of interest.

AUTHOR CONTRIBUTIONS

Conception and design: Motoki Iwasaki, Manami Inoue, Tetsuya Otani, Shizuka Sasazuki, Seiichiro Yamamoto, Shoichiro Tsugane
Financial support: Motoki Iwasaki, Shoichiro Tsugane
Administrative support: Motoki Iwasaki, Manami Inoue, Tetsuya Otani, Shizuka Sasazuki, Norie Kurahashi, Tsutomu Miura, Seiichiro Yamamoto
Collection and assembly of data: Motoki Iwasaki, Manami Inoue, Tetsuya Otani, Shizuka Sasazuki, Norie Kurahashi, Seiichiro Yamamoto, Shoichiro Tsugane
Data analysis and interpretation: Motoki Iwasaki, Manami Inoue, Tetsuya Otani, Shizuka Sasazuki, Norie Kurahashi, Tsutomu Miura, Seiichiro Yamamoto, Shoichiro Tsugane
Manuscript writing: Motoki Iwasaki
Final approval of manuscript: Motoki Iwasaki, Manami Inoue, Tetsuya Otani, Shizuka Sasazuki, Norie Kurahashi, Tsutomu Miura, Seiichiro Yamamoto, Shoichiro Tsugane

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Appendix

The Appendix is included in the full-text version of this article, available online at www.jco.org. It is not included in the PDF version (via Adobe® Reader®).

Isoflavone, polymorphisms in estrogen receptor genes and breast cancer risk in case-control studies in Japanese, Japanese Brazilians and non-Japanese Brazilians

Motoki Iwasaki,[1,11] Gerson Shigeaki Hamada,[2] Inês Nobuko Nishimoto,[3] Mario Mourão Netto,[4] Juvenal Motola Jr,[5] Fábio Martins Laginha,[5] Yoshio Kasuga,[6] Shiro Yokoyama,[7] Hiroshi Onuma,[7] Hideki Nishimura,[8] Ritsu Kusama,[9] Minatsu Kobayashi,[1] Junko Ishihara,[1] Seiichiro Yamamoto,[10] Tomoyuki Hanaoka[1] and Shoichiro Tsugane[1]

[1]Epidemiology and Prevention Division, Research Center for Cancer Prevention and Screening, National Cancer Center, Tokyo 104-0045, Japan; [2]Nikkei Disease Prevention Center, São Paulo 04120-060, Brazil; [3]Statistical Section/Head and Neck Surgery and Otorhinolaryngology Department, Hospital A.C. Camargo, São Paulo 01509-900, Brazil; [4]Breast Surgery Department, Hospital A.C. Camargo, São Paulo 01509-900, Brazil; [5]Department of Breast Surgery, Hospital Pérola Byington, São Paulo 01317-000, Brazil; [6]Department of Surgery, Nagano Matsushiro General Hospital, Nagano 381-1231, Japan; [7]Department of Breast and Thyroid Surgery, Nagano Red Cross Hospital, Nagano 380-8582, Japan; [8]Department of Surgery, Nagano Municipal Hospital, Nagano 381-8551, Japan; [9]Department of Surgery, Nagano Hokushin General Hospital, Nagano 383-8505, Japan; [10]Cancer Information Services and Surveillance Division, Center for Cancer Control and Information Services, National Cancer Center, Tokyo 104-0045, Japan

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Epidemiologic studies have shown an inverse association between isoflavones and breast cancer risk. Because isoflavones bind estrogen receptors, we hypothesized that polymorphisms in the estrogen receptor genes might modify the association between isoflavone intake and breast cancer risk. 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 among medical checkup examinees in Nagano, Japan, and from cancer-free patients in São Paulo, Brazil. A total of 846 pairs (388 Japanese, 79 Japanese Brazilians and 379 non-Japanese Brazilians) completed validated food frequency questionnaires, and provided blood samples. Five single nucleotide polymorphisms in the estrogen receptor alpha (rs9340799, rs1913474, and rs2234693) and beta (rs4986938 and rs1256049) genes were genotyped. We found no consistent association between the five single nucleotide polymorphisms and breast cancer risk among the three populations. In analyses of combinations of isoflavone intake and single nucleotide polymorphisms, an inverse association between intake and risk was limited to women with the GG genotype of the rs4986938 polymorphism for postmenopausal Japanese (odds ratio for highest *versus* lowest tertile = 0.47; *P* for trend = 0.01), Japanese Brazilians (odds ratio for highest *versus* lowest median = 0.31) and non-Japanese Brazilians (odds ratio for consumers *versus* non-consumers = 0.37) (*P* for interaction = 0.11, 0.08, and 0.21, respectively). We found no remarkable difference for the other four polymorphisms. Our findings suggest that polymorphisms in the estrogen receptor beta gene may modify the association between isoflavone intake and breast cancer risk. (*Cancer Sci* 2009; 100: 927–933)

Soy foods are a traditional staple dish in Asian countries. They are a primary source of isoflavones such as genistein and daidzein, which are classified as phytoestrogens. Because breast cancer risk is substantially lower in Asian than Western countries,[1] the contribution of a high isoflavone intake to low breast cancer risk has been hypothesized.[2] A meta-analysis supported this hypothesis and found a small decrease in breast cancer risk with higher soy intake.[3] On the other hand, a more recent meta-analysis indicated that risk reduction was limited to Asian populations.[4] This discrepancy might reflect differences in exposure levels and genetic factors between Asian and Western populations.

Several mechanisms by which isoflavones may reduce the risk of breast cancer have been proposed.[5,6] The most prominent and thoroughly investigated are those mediated via estrogen receptors, which arise due to the similarity in chemical structures between isoflavones and human estrogen hormone, and the consequent binding affinity of isoflavones for estrogen receptors.[6,7] Isoflavones can therefore act as estrogen agonists and antagonists competing for estradiol at the receptor complex,[5] suggesting in turn that isoflavones might interact with estrogen receptor genes in the development of breast cancer. However, the possible joint effect of isoflavone intake and polymorphisms in the estrogen receptor genes on the risk of breast cancer has not been investigated.

Here, we conducted hospital-based case-control studies in Nagano, Japan and São Paulo, Brazil, targeting three populations with a substantially different intake of isoflavones and distribution of polymorphisms in the estrogen receptor genes: Japanese living in Japan, Japanese Brazilians living in São Paulo, and non-Japanese Brazilians living in São Paulo. In a previous report, we found a non-significant inverse association between isoflavone intake and the risk of breast cancer in postmenopausal Japanese women but a statistically significant inverse association in Japanese Brazilians and non-Japanese Brazilians.[8] Based on this finding, the present study tested the hypothesis that polymorphisms in estrogen receptor genes may modify the association between isoflavone intake and breast cancer risk.

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. Patients with cancer were recruited between 2001 and 2005 at four hospitals in Nagano, and between 2001 and 2006 at eight hospitals in São Paulo, totaling 405 patients (98%) in Nagano, and 83 Japanese Brazilians (91%) and 389 non-

[11]To whom correspondence should be addressed. E-mail: moiwasak@ncc.go.jp
Abbreviations: CI, confidence interval; CYP17, cytochrome P450c17α; CYP19, aromatase; CYP2E1, cytochrome P450 2E1; ESR1, estrogen receptor alpha; ESR2, estrogen receptor beta; FFQ, food-frequency questionnaire; NAT2, N-acetyltransferase 2; OR, odds ratio; SNP, single-nucleotide polymorphism.

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Table 1. Single-nucleotide polymorphisms in estrogen receptor genes and their allele frequency

Gene	SNP rs number	Synonym	Region	Major/minor allele	Minor allele frequency among control groups		
					Japanese living in Nagano, Japan	Japanese Brazilians living in São Paulo, Brazil	Non-Japanese Brazilians living in São Paulo, Brazil
Estrogen receptor alpha gene	rs9340799	XbaI	intron 1	A/G	0.19	0.20	0.31
	rs1913474		intron 3	C/T	0.48	0.48	0.21
	rs2234693	PvuII	intron 1	T/C	0.45	0.45	0.42
Estrogen receptor beta gene	rs4986938	AuII	3'-UTR	G/A	0.14	0.13	0.33
	rs1256049	RsaI	exon 6	G/A	0.30	0.20	0.05

SNP, single-nucleotide polymorphism.

Japanese Brazilians (99%) in São Paulo. In the Nagano study, 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. Among potential controls, one examinee refused to participate and two refused to provide blood samples. Eventually, 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 patient with cancer by age (within 5 years) and ethnicity. Among potential controls, 22 patients refused to participate (participation rate = 96%). Eventually, 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 Comissão Nacional de Ética em Pesquisa (CONEP), Brasília, Brazil and by the institutional review board of the National Cancer Center, Tokyo, Japan.

Questionnaire. Participants in Nagano were asked to complete a self-administered questionnaire, while those in São Paulo were interviewed by trained interviewers using a structured questionnaire. The two questionnaires contained 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 semiquantitative food frequency questionnaire (FFQ) (136 items for the Japanese version and 118 items for the Brazilian version), which was developed and validated in each population.[9–11] 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. 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.[12,13] 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 for the Japanese version,[14] and the United States Department of Agriculture (USDA) food composition tables for the Brazilian version.[15] For some Japanese-specific foods in the Brazilian version, the Japanese Standard Tables of Food Composition[14] 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 by comparing the estimated intake according to the FFQ to that in four consecutive seven-day dietary records, one conducted in each of the four seasons. Spearman's correlation coefficients between energy-adjusted genistein and daidzein intake estimated from the FFQ and from dietary records to be 0.59 for genistein and 0.60 for daidzein.[10] For the Brazilian version, the validity of isoflavone intake estimated from the FFQ was evaluated in a subsample of the control group of this case-control study by comparing the estimated intake according to the FFQ to that in two consecutive four-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.[11]

Genotyping. Genomic DNA samples were extracted from the peripheral blood using FlexiGene® DNA kits (Qiagen K.K., Tokyo, Japan) according to the manufacturer's protocol. We selected five single nucleotide polymorphisms (SNPs) in the estrogen receptor alpha (*ESR1*) gene (rs9340799, rs1913474, and rs2234693) and estrogen receptor beta (*ESR2*) gene (rs4986938 and rs1256049), which were the most frequently studied SNP in relation to breast cancer risk.[16–20] Genotyping of the five SNPs was performed by a commercial laboratory (Genetic Laboratory, Inc., Sapporo, Japan) using TaqMan® SNP Genotyping Assays (Applied Biosystems, Foster City, CA, USA) (Table 1). Patients with cancer and matched controls were analyzed in the same well by laboratory personnel unaware of the case-control status. For quality control assessment, we genotyped six SNPs of four genes (N-acetyltransferase 2 [*NAT2*], cytochrome P450c17α [*CYP17*], aromatase [*CYP19*], and cytochrome P450 2E1 [*CYP2E1*]) in our laboratory using about 24% of the samples in the present study. However, SNPs used in the present study were not included. The concordance rates between Genetic Laboratory Inc. and our laboratory varied between 97.6 and 99.5% among the six SNPs.

Statistical analysis. Comparison of baseline characteristics between cases and controls was evaluated by the Mantel–Haenszel test using matched-pair strata in each population. Genotype frequencies were tested for deviation from the Hardy–Weinberg equilibrium with the χ^2-test. 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, SNPs, and the joint effect between isoflavone intake and genotypes. An unconditional logistic regression model was used for stratified analyses according to menopausal status. Linear trends for ORs were tested in the logistic regression model using the exposure categories as ordinal variables. Tests for the interaction were performed based on the difference between two likelihood ratios of the models with and without the interaction terms between isoflavone intake and the SNP of interest. Adjustments were made for the following variables, selected mainly on the basis of comparison of baseline characteristics between patients with cancer and controls, 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

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Table 2. Odds ratios and 95% confidence intervals of breast cancer according to polymorphisms in estrogen receptor genes

	Japanese living in Nagano, Japan				Japanese Brazilians living in São Paulo, Brazil				Non-Japanese Brazilians living in São Paulo, Brazil			
	No.		OR†	95% CI	No.		OR†	95% CI	No.		OR†	95% CI
	Case	Control			Case	Control			Case	Control		
Estrogen receptor alpha gene (rs9340799)												
AA	273	256	1		54	50	1		161	182	1	
AG	103	119	0.68	(0.45–1.02)	22	26	0.75	(0.31–1.84)	175	161	1.16	(0.84–1.59)
GG	12	13	0.75	(0.28–1.98)	3	3	0.68	(0.10–4.57)	43	36	1.27	(0.78–2.07)
AG + GG	115	132	0.69	(0.47–1.02)	25	29	0.74	(0.31–1.79)	218	197	1.18	(0.88–1.59)
Estrogen receptor alpha gene (rs1913474)												
CC	100	113	1		25	24	1		237	239	1	
CT	192	176	1.19	(0.81–1.76)	39	34	1.24	(0.55–2.81)	127	122	1.09	(0.80–1.49)
TT	96	99	1.08	(0.70–1.66)	15	21	0.79	(0.28–2.20)	14	18	0.80	(0.38–1.67)
CT + TT	288	275	1.15	(0.80–1.64)	54	55	1.07	(0.51–2.27)	141	140	1.05	(0.78–1.42)
Estrogen receptor alpha gene (rs2234693)												
TT	144	115	1		25	22	1		107	122	1	
TC	180	196	**0.70**	**(0.49–0.995)**	39	43	0.66	(0.29–1.47)	187	194	0.99	(0.68–1.43)
CC	64	77	0.64	(0.40–1.02)	15	14	0.93	(0.31–2.86)	85	63	1.51	(0.98–2.31)
TC + CC	244	273	**0.68**	**(0.49–0.96)**	54	57	0.71	(0.32–1.54)	272	257	1.15	(0.83–1.61)
Estrogen receptor beta gene (rs4986938)												
GG	289	281	1		59	60	1		169	176	1	
GA	94	102	0.88	(0.59–1.31)	17	17	1.32	(0.53–3.31)	163	154	1.09	(0.78–1.51)
AA	5	5	1.53	(0.39–6.07)	3	2	0.71	(0.09–5.57)	47	49	0.93	(0.59–1.47)
GA + AA	99	107	0.91	(0.62–1.34)	20	19	1.22	(0.51–2.93)	210	203	1.05	(0.77–1.42)
Estrogen receptor beta gene (rs1256049)												
GG	203	182	1		47	48	1		342	345	1	
GA	161	178	0.79	(0.56–1.10)	26	30	0.95	(0.46–1.98)	36	32	1.21	(0.71–2.04)
AA	24	28	0.84	(0.44–1.60)	6	1	4.80	(0.50–46.19)	1	2	0.54	(0.04–6.53)
GA + AA	185	206	0.79	(0.57–1.09)	32	31	1.04	(0.50–2.13)	37	34	1.16	(0.70–1.94)

†Conditional model adjusting for menopausal status (premenopausal, postmenopausal), 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). ORs and 95% CIs with statistical significance are written in bold letter. CIs, confidence intervals; OR, odds ratio.

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 version 9.1 software (SAS Institute, Inc., Cary, NC, USA).

Results

We excluded subjects who reported extremely low or high total energy intake (<500 or ≥4000 kCal) or had no DNA sample, leaving 388 pairs of Japanese, 79 pairs of Japanese Brazilians and 379 pairs of non-Japanese Brazilians for inclusion in the present analyses.

Characteristics of patients with cancer and controls are shown in a previous report (data not shown in table).[8] For Japanese women, the proportion of premenopausal women, current smokers, and vitamin supplement users was higher in cases than in controls, and patients with cancer tended to have a family history of breast cancer and history of benign breast disease. Patients with cancer were less likely than controls to breast-feed, be physically active and eat vegetables. For Japanese Brazilians, patients with cancer 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 patients with cancer than controls, while the proportion of physically active women and vitamin supplement users was lower. Isoflavone intake substantially varied among populations, with mean intakes in control subjects of 46.2 mg/day for Japanese, 23.5 mg/day for Japanese Brazilians, and 4.4 mg/day for non-Japanese Brazilians.

The distributions of SNPs in the *ESR1* gene (rs9340799, rs1913474 and rs2234693) and *ESR2* gene (rs4986938 and rs1256049) are shown in Tables 1 and 2. No deviation from the Hardy–Weinberg equilibrium was observed among the controls in any population. The prevalence of the minor allele in the rs9340799 and rs4986938 polymorphisms was lower in the control group of Japanese and Japanese Brazilians than in that of non-Japanese Brazilians, while that of the minor allele in the rs1913474 and rs1256049 polymorphisms was higher in the control group of Japanese and Japanese Brazilians. We found a decreased risk of breast cancer among Japanese women with at least one minor allele of the rs9340799 or rs2234693 polymorphism in comparison with those with the major allele homozygote, but not among Japanese Brazilian and non-Japanese Brazilian women. This decrease was statistically significant for the rs2234693 polymorphism but not for the rs9340799 polymorphism. Stratified analyses by menopausal status showed that this decreased risk occurred primarily among postmenopausal Japanese for both SNPs (data not shown). In contrast, no association was observed for the rs1913474, rs4986938, or rs1256049 polymorphisms in the three populations, regardless of menopausal status.

Analyses of combinations of isoflavone intake and the rs4986938 polymorphism in the *ESR2* gene revealed that the risk of breast cancer significantly decreased with increasing isoflavone intake only among women with the GG genotype among postmenopausal Japanese (OR for highest *versus* lowest tertile = 0.47; 95%CI 0.27–0.84; *P* for trend = 0.01), Japanese Brazilians (OR for highest *versus* lowest median = 0.31; 95%CI 0.12–0.78), and non-Japanese Brazilians (OR for consumers *versus* non-consumers = 0.37; 95%CI

Table 3. Odds ratios and 95% confidence intervals of breast cancer for combinations of dietary intake of isoflavones and polymorphisms in estrogen receptor genes among Japanese

	All subjects				Premenopausal women				Postmenopausal women			
	Isoflavone intake (mg/day), tertile category			*P* for trend	Isoflavone intake (mg/day), tertile category			*P* for trend	Isoflavone intake (mg/day), tertile category			*P* for trend
	1	2	3		1	2	3		1	2	3	
Estrogen receptor alpha gene (rs9340799)												
AA												
No.†	109/83	76/90	88/83		54/41	30/31	33/19		55/42	46/59	55/64	
OR‡	1	0.73	0.78	0.32	1	0.68	1.13	0.96	1	0.75	0.64	0.15
(95% CI)		(0.45–1.18)	(0.47–1.29)			(0.34–1.35)	(0.53–2.39)			(0.41–1.34)	(0.36–1.15)	
AG + GG												
No.†	42/46	42/39	31/47		25/25	22/12	12/7		17/21	20/27	19/40	
OR‡	**0.52**	0.68	0.51	0.75	0.64	1.38	1.13	0.54	0.59	0.56	**0.38**	0.15
(95% CI)	**(0.27–0.99)**	(0.37–1.24)	(0.26–1.01)		(0.31–1.35)	(0.59–3.23)	(0.39–3.30)		(0.26–1.32)	(0.26–1.20)	**(0.18–0.79)**	
	P for interaction = 0.39				*P* for interaction = 0.15				*P* for interaction = 0.87			
Estrogen receptor alpha gene (rs1913474)												
CC												
No.†	41/38	32/42	27/33		20/16	16/12	13/4		21/22	16/30	14/29	
OR‡	1	0.68	0.76	0.62	1	1.15	2.39	0.08	1	0.60	0.47	0.09
(95% CI)		(0.34–1.36)	(0.37–1.59)			(0.40–3.26)	(0.61–9.30)			(0.24–1.47)	(0.18–1.21)	
CT + TT												
No.†	110/91	86/87	92/97		59/50	36/31	32/22		51/41	50/56	60/75	
OR‡	0.97	0.97	0.84	0.33	0.91	0.86	0.97	0.99	1.20	1.08	0.80	0.14
(95% CI)	(0.54–1.74)	(0.55–1.72)	(0.45–1.55)		(0.41–2.02)	(0.37–2.04)	(0.39–2.44)		(0.56–2.59)	(0.51–2.29)	(0.38–1.68)	
	P for interaction = 0.69				*P* for interaction = 0.58				*P* for interaction = 0.73			
Estrogen receptor alpha gene (rs2234693)												
TT												
No.†	58/36	38/41	48/38		33/21	12/16	21/11		25/15	26/25	27/27	
OR‡	1	0.55	0.68	0.54	1	0.41	1.15	0.77	1	0.79	0.58	0.28
(95% CI)		(0.26–1.16)	(0.32–1.43)			(0.15–1.12)	(0.43–3.10)			(0.32–1.92)	(0.24–1.40)	
TC + CC												
No.†	93/93	80/88	71/92		46/45	40/27	24/15		47/48	40/61	47/77	
OR‡	**0.51**	**0.52**	**0.42**	0.46	0.64	0.99	0.86	0.39	0.54	**0.42**	**0.35**	0.14
(95% CI)	**(0.28–0.96)**	**(0.28–0.98)**	**(0.21–0.82)**		(0.31–1.34)	(0.45–2.16)	(0.35–2.15)		(0.24–1.21)	**(0.19–0.92)**	**(0.16–0.76)**	
	P for interaction = 0.37				*P* for interaction = 0.08				P for interaction = 0.97			
Estrogen receptor beta gene (rs4986938)												
GG												
No.†	115/86	88/96	86/99		57/46	39/32	32/21		58/40	49/64	54/78	
OR‡	1	0.74	0.65	0.06	1	0.96	1.03	0.94	1	0.60	**0.47**	**0.01**
(95% CI)		(0.47–1.16)	(0.39–1.07)			(0.51–1.83)	(0.49–2.15)			(0.33–1.07)	**(0.27–0.84)**	
GA + AA												
No.†	36/43	30/33	33/31		22/20	13/11	13/5		14/23	17/22	20/26	
OR‡	0.57	0.78	0.90	0.23	0.80	0.91	1.99	0.20	0.47	0.80	0.62	0.49
(95% CI)	(0.31–1.08)	(0.40–1.50)	(0.45–1.82)		(0.37–1.72)	(0.35–2.34)	(0.62–6.46)		(0.21–1.06)	(0.36–1.75)	(0.28–1.35)	
	P for interaction = 0.17				*P* for interaction = 0.48				*P* for interaction = 0.11			
Estrogen receptor beta gene (rs1256049)												
GG												
No.†	85/62	59/62	59/58		43/32	28/20	23/12		42/30	31/42	36/46	
OR‡	1	0.74	0.82	0.16	1	1.05	0.98	0.80	1	0.56	0.51	0.08
(95% CI)		(0.43–1.27)	(0.46–1.48)			(0.49–2.27)	(0.39–2.47)			(0.28–1.13)	(0.26–1.01)	
GA + AA												
No.†	66/67	59/67	60/72		36/34	24/23	22/14		30/33	35/44	38/58	
OR‡	0.70	0.74	0.61	0.93	0.79	0.78	1.31	0.20	0.50	0.60	**0.41**	0.35
(95% CI)	(0.41–1.19)	(0.43–1.27)	(0.35–1.07)		(0.39–1.58)	(0.36–1.69)	(0.55–3.08)		(0.24–1.03)	(0.31–1.18)	**(0.21–0.80)**	
	P for interaction = 0.63				*P* for interaction = 0.65				*P* for interaction = 0.31			

†No. of patients with cancer/No. of controls.
‡Conditional model adjusting for menopausal status (premenopausal, postmenopausal), number of births (0, 1, 2, 3, 4, 5+), family history of breast cancer (yes, no), smoking status (never, past, current smoker), 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). For stratified analyses according to menopausal status, an unconditional model adjusting for age, area, number of births (0, 1, 2, 3, 4, 5+), family history of breast cancer (yes, no), smoking status (never, past, current smoker), 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). ORs and 95% CIs with statistical significance are written in bold letter. CIs, confidence intervals; OR, odds ratio.

0.16–0.85) (*P* for interaction = 0.11, 0.08 and 0.21, respectively) (Tables 3 and 4). Moreover, we found no remarkable difference in the association between isoflavone intake and breast cancer risk by the four other polymorphisms.

Discussion

In these case-control studies of Japanese, Japanese Brazilians, and non-Japanese Brazilians, we found that a statistically significant

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Table 4. Odds ratios and 95% confidence intervals of breast cancer for combinations of dietary intake of isoflavones and polymorphisms in estrogen receptor genes among Japanese Brazilian and non-Japanese Brazilian subjects

	Japanese Brazilians living in São Paulo, Brazil		Non-Japanese Brazilians living in São Paulo, Brazil	
	Isoflavone intake (mg/day), median category		Isoflavone intake (mg/day)	
	1	2	Non-consumers	Consumers
Estrogen receptor alpha gene (rs9340799)				
AA				
No.†	31/21	23/29	145/157	16/25
OR‡	1	**0.36**	1	0.68
(95% CI)		**(0.14–0.95)**		(0.32–1.43)
AG + GG				
No.†	15/18	10/11	198/161	20/36
OR‡	0.44	0.34	1.23	0.61
(95% CI)	(0.14–1.45)	(0.09–1.32)	(0.89–1.68)	(0.33–1.13)
	P for interaction = 0.36		*P* for interaction = 0.52	
Estrogen receptor alpha gene (rs1913474)				
CC				
No.†	13/12	12/12	213/204	24/35
OR‡	1	0.76	1	0.65
(95% CI)		(0.18–3.18)		(0.36–1.19)
CT + TT				
No.†	33/27	21/28	129/114	12/26
OR‡	1.25	0.55	1.13	0.49
(95% CI)	(0.42–3.72)	(0.17–1.78)	(0.82–1.56)	(0.24–1.01)
	P for interaction = 0.52		*P* for interaction = 0.40	
Estrogen receptor alpha gene (rs2234693)				
TT				
No.†	17/12	8/10	97/106	10/16
OR‡	1	0.41	1	0.57
(95% CI)		(0.10–1.65)		(0.22–1.47)
TC + CC				
No.†	29/27	25/30	246/212	26/45
OR‡	0.65	0.36	1.20	0.65
(95% CI)	(0.23–1.84)	(0.12–1.08)	(0.84–1.71)	(0.37–1.15)
	P for interaction = 0.71		*P* for interaction = 0.94	
Estrogen receptor beta gene (rs4986938)				
GG				
No.†	38/30	21/30	156/148	13/28
OR‡	1	**0.31**	1	**0.37**
(95% CI)		**(0.12–0.78)**		**(0.16–0.85)**
GA + AA				
No.†	8/9	12/10	156/170	23/33
OR‡	0.62	0.97	0.97	0.68
(95% CI)	(0.16–2.35)	(0.31–3.01)	(0.70–1.35)	(0.37–1.24)
	P for interaction = 0.08		*P* for interaction = 0.21	
Estrogen receptor beta gene (rs1256049)				
GG				
No.†	27/23	20/25	308/286	34/59
OR‡	1	0.49	1	**0.55**
(95% CI)		(0.21–1.17)		**(0.35–0.90)**
GA + AA				
No.†	19/16	13/15	35/32	2/2
OR‡	0.97	0.53	1.10	0.84
(95% CI)	(0.36–2.58)	(0.18–1.58)	(0.64–1.87)	(0.10–6.97)
	P for interaction = 0.89		*P* for interaction = 0.78	

†No. of patients with cancer/No. of controls.
‡Conditional model adjusting for menopausal status (premenopausal, postmenopausal), 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). ORs and 95% CIs with statistical significance are written in bold letter. CIs, confidence intervals; OR, odds ratio.

inverse association between isoflavone intake and breast cancer risk appeared only among women with the GG genotype of the rs4986938 polymorphism in the *ESR2* gene, but the interaction was not statistically significant. Our findings support the hypothesis that polymorphisms in the *ESR2* gene may modify the association between isoflavone intake and breast cancer risk.

To date, many studies investigating the possible effect of SNPs in the *ESR2* gene on breast cancer risk have focused on the

rs4986938 and rs1256049 polymorphisms, although their functional importance has yet to be clarified. Here, we found no association between either SNP and the risk of breast cancer, which is in general agreement with most previous studies.[16,17] In contrast, we did see an inverse association between isoflavone intake and breast cancer risk with the rs4986938 polymorphism in three populations, but only among women with the GG genotype. We also saw a suggestive interaction in the case-control studies of Japanese and Japanese Brazilians but not in the case-control study of non-Japanese Brazilians. Although the reason for the inconsistency in interactions among populations remains unclear, it might reflect the amount of intake, on the basis that the findings were relatively consistent among the populations with a high intake (Japanese and Japanese Brazilians). Moreover, the prevalence of the GG genotype of the rs4986938 polymorphism among the control group was higher in Japanese (72.4%) and Japanese Brazilians (75.9%) than in non-Japanese Brazilians (46.4%). This might partly explain the previous inconsistencies in results for isoflavone exposure and breast cancer risk between Asian and Western populations.[4]

To our knowledge, only two studies have investigated interactions between phytoestrogen exposure and polymorphisms in the *ESR2* gene in the risk of hormone-related diseases.[21,22] Hedelin *et al.* reported a significant interaction between phytoestrogen intake and a promoter SNP in the *ESR2* gene (rs2987983) in the risk of prostate cancer in a population-based case-control study in Sweden.[22] Tsuchiya *et al.* reported a significant interaction between urinary genistein level and RsaI polymorphism in the *ESR2* gene in the risk of advanced endometriosis among infertile Japanese women.[21] These findings suggest that isoflavones may reduce the risk of hormone-related diseases via a mechanism that involves estrogen receptor beta. Considering that functional data are not presently available, our finding suggests that the rs4986938 polymorphism, or some other genetic variants in strong linkage disequilibrium with this SNP, modify the protective effect of isoflavones on breast cancer. In this regard, we provide further evidence for a role of isoflavones in the development of breast cancer.

We found a decreased risk of breast cancer among Japanese women with at least one minor allele of the rs9340799 or rs2234693 polymorphism in comparison with those with the major allele homozygote. Although these are the most frequently studied SNPs, results have been inconsistent.[18–20] Most studies have shown no association between the rs2234693 polymorphism and breast cancer risk.[18–20] On the other hand, several but not all studies have reported that the G allele of the rs9340799 polymorphism was associated with a decreased risk of breast cancer,[18,20] which is consistent with our findings in Japanese women. Since we failed to observe an overall consistency of findings in our three populations, however, our findings in Japanese women might be merely due to chance.

Although interactions between phytoestrogen exposure and polymorphisms in the *ESR1* gene in the risk of breast cancer have not been investigated, we are aware of two studies examining interactions on circulating sex hormone levels.[23,24] In their study of 125 postmenopausal women in the European Prospective Investigation of Cancer and Nutrition–Norfolk cohort, Low *et al.* reported that urinary and serum isoflavones were negatively correlated with plasma estradiol among women with the CC genotype for PvuII polymorphism in the *ESR1* gene, but not those with other genotypes.[23] Moreover, they reported a significant interaction between urinary lignans and rs9340835 polymorphism in the *ESR1* gene, affecting plasma estrone levels in a cross-sectional study of 1988 healthy postmenopausal women from the same cohort.[24] Although these studies imply the presence of gene–nutrient interaction, we found no remarkable difference in the association between isoflavone intake and breast cancer risk by polymorphisms in the *ESR1* gene. Further studies based on a comprehensive evaluation of this gene would clarify this gene–nutrient interaction.

Our study has methodological advantages over studies conducted previously. First, and unique to this study, we assessed gene–nutrient interactions using three populations with substantially different isoflavone intakes and allele frequencies of SNPs. For example, isoflavone intake differed considerably among the three populations, with median levels (interquartile range) in the control group of (mg/day) 40.7 (25.8–61.4) among Japanese, 13.4 (7.9–31.1) among Japanese Brazilians, and 0 (0–0) among non-Japanese Brazilians. In addition, allele frequency also differed among the populations, such as that of the G allele of the rs4986938 polymorphism in the *ESR2* gene, at 0.86 for Japanese, 0.87 for Japanese Brazilians, and 0.67 for non-Japanese Brazilians. Second, the overall consistency of findings in the three populations could allow the results to be more generalized than those from a single population.

Several limitations of the study also warrant mention. First, dietary intake of isoflavones was assessed after the diagnosis of breast cancer, and therefore, is sensitive to recall bias. Second, although the substantially high participation rates among both eligible patients with cancer 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 those of the general population due to health consciousness or disease, might have lead to selection bias. For example, isoflavone intake was higher among women aged 50–69 years in the control group of the Nagano study (median intake = 46.3 mg/day) than in participants aged 50–69 years living in Nagano in the 10-year follow-up survey of the Japan Public Health Center-based Prospective Study (median intake = 38.8 mg/day), which used a similar FFQ and had a high response rate. Third, the evaluation of gene–nutrient interactions was performed in a relatively small number of patients with cancer. The interpretability of our results might therefore be limited.

Allowing for these methodological issues, we found a suggestive interaction between isoflavone intake and the rs4986938 polymorphism of the *ESR2* gene in the risk of breast cancer in case-control studies of Japanese and Japanese Brazilians. Our findings support the hypothesis that polymorphisms in the *ESR2* gene may modify the association between isoflavone intake and breast cancer risk. Further, they provide additional evidence that the mechanisms by which isoflavones may reduce the risk of breast cancer might involve estrogen receptor beta.

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EPIDEMIOLOGY

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

Motoki Iwasaki · Gerson Shigeaki Hamada · Ines Nobuko Nishimoto · Mario Mourão Netto · Juvenal Motola Jr. · Fábio Martins Laginha · Yoshio Kasuga · Shiro Yokoyama · Hiroshi Onuma · Hideki Nishimura · Ritsu Kusama · Minatsu Kobayashi · Junko Ishihara · Seiichiro Yamamoto · Tomoyuki Hanaoka · Shoichiro Tsugane

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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

M. Iwasaki (✉) · M. Kobayashi · J. Ishihara · T. Hanaoka · S. Tsugane
Epidemiology and Prevention Division, Research Center for Cancer Prevention and Screening, National Cancer Center, 5-1-1 Tsukiji, Chuo-ku, Tokyo 104-0045, Japan
e-mail: moiwasak@ncc.go.jp

G. S. Hamada
Nikkei Disease Prevention Center, São Paulo, Brazil

I. N. Nishimoto
Statistical Section/Head and Neck Surgery and Otorhinolaryngology Department, Hospital A.C. Camargo, São Paulo, Brazil

M. M. Netto
Breast Surgery Department, Hospital A.C. Camargo, São Paulo, Brazil

J. Motola Jr. · F. M. Laginha
Department of Breast Surgery, Hospital Pérola Byington, São Paulo, Brazil

Y. Kasuga
Department of Surgery, Nagano Matsushiro General Hospital, Nagano, Japan

S. Yokoyama · H. Onuma
Department of Breast and Thyroid Surgery, Nagano Red Cross Hospital, Nagano, Japan

H. Nishimura
Department of Surgery, Nagano Municipal Hospital, Nagano, Japan

R. Kusama
Department of Surgery, Nagano Hokushin General Hospital, Nagano, Japan

S. Yamamoto
Cancer Information Services and Surveillance Division, Center for Cancer Control and Information Services, National Cancer Center, Tokyo, Japan

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