members of our Study Group 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 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, and based on death certificates only in 0.7%. The mortality/incidence ratio was 0.14.

For each case, two controls were selected using incidence density sampling from subjects who were not diagnosed with breast cancer during the follow-up period when the case was diagnosed. Control selection was done without reference to the incidence of other cancer sites. Controls were matched with each case 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 h), time since last meal at blood collection (within 3 h), and baseline menopausal status.

Laboratory assay

Plasma EGC, EC, EGCG, and ECG were analyzed using high-performance liquid chromatography with a coulometric array detector in accordance with the modified methods of Lee et al. [15, 16]. Concentrations of tea polyphenols 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 tea polyphenols revealed a linearity range 0–0.5 μg/ml, with correlation coefficient values > 0.998. Voltammetric response for the standard solution displayed coefficients of variation of 10% for intra- and 11% for inter-day variation. Recovery rates of tea polyphenols in plasma samples ranged between approximately 63 and 90% (EGC, 90%; EC, 89%; EGCG, 66%; and ECG, 63%). Detection limits were 1.4 ng/ml for EGCG, 1.6 ng/ml for EGC, 1.8 ng/ml for ECG, and 0.3 ng/ml for EC. Laboratory personnel were blinded to case–control status when performing the analyses.

Statistical analysis

Comparison of baseline characteristics between cases and controls was evaluated by the Mantel–Haenszel test using matched-set strata. Spearman's correlation coefficients were calculated between plasma levels and green tea intake assessed using the food frequency questionnaire (FFQ) among control subjects. Using a conditional logistic regression model, we calculated odds ratios (ORs) and 95% confidence intervals (CIs) of breast cancer for plasma tea polyphenol levels categorized into three groups based on the control distribution, namely, under the detection limit, and under or over the median level among detected samples. The following variables were adjusted for as potential confounders: 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), body mass index (BMI) (continuous), alcohol intake (non-drinkers, occasional drinkers, 50 (g/week), 50–100 (g/week), 100+ (g/week) among regular drinkers (ethanol)). Linear trends for ORs were tested in the conditional logistic regression model using the exposure categories as ordinal variables. To perform stratified analyses according to menopausal status, levels of tea polyphenols in the plasma were dichotomized into the "detected" and "not detected" categories. Since previous studies have suggested effect modification of green tea intake by dietary factors [10, 13], we further performed stratified analyses by plasma genistein level, and dietary isoflavone and folate intake. 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

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

The proportion of subjects in whom tea polyphenols were not detected was 69% for EGC, 76% for EC, 80% for EGCG, and 81% for ECG. Correlation coefficients between plasma levels and green tea intake were low, at 0.23 for EGC, 0.16 for EC, 0.18 for EGCG, and 0.17 for ECG. We found no statistically significant association between plasma tea polyphenol level and the risk of breast cancer (Table 2). Adjusted ORs for the highest versus lowest group were 0.90 (95% CI 0.42–1.96; P for trend = 0.98) for EGC, 0.95 (95% CI 0.43–2.08; P for trend = 0.86) for EC, 1.21 (95% CI 0.52-2.80; P for trend = 0.53) for EGCG, and 1.75 (95% CI 0.81–3.78; P for trend = 0.15) for ECG. In addition to each tea polyphenol, total plasma tea polyphenol level, defined as the sum of plasma EGC, EC, EGCG, and ECG levels, was not associated with the risk of breast cancer (data not shown). No substantial change was seen after further adjustment for other potential confounders, namely family history of breast cancer, use of exogenous female hormones, leisure-time physical activity, smoking habit, and dietary intake of meat, fish, vegetables, fruit, energy and isoflavones (data not shown). Further,

Table 1 Characteristics of case and matched control subjects at baseline

	Case (n = 144)	Control (n = 288)	P[a]
Age (year), mean (SE)	52 (0.6)	52 (0.4)	–
Family history of breast cancer, n (%)	2 (1)	2 (0.7)	0.48
Premenopausal women, n (%)	59 (42)	118 (42)	–
Age at menopause (year), mean (SE)[b]	50 (0.4)	50 (0.3)	0.76
Age at menarche (year), mean (SE)[b]	15 (0.1)	15 (0.1)	0.33
Number of births, mean (SE)[b]	2 (0.1)	3 (0.1)	0.01
Age at first birth (year), mean (SE)[b]	26 (0.3)	25 (0.2)	0.22
Use of exogenous female hormones (current use), n (%)	4 (3)	2 (0.8)	0.10
Height (cm), mean (SE)[b]	152 (0.5)	151 (0.3)	0.70
Body mass index (kg/m^2), mean (SE)[b]	23 (0.3)	24 (0.2)	0.49
Smoking (current smoker), n (%)	5 (3)	17 (6)	0.23
Alcohol drinking (regular drinker), n (%)	18 (13)	26 (9)	0.28
Leisure-time physical activity (≥once per week), n (%)	30 (21)	57 (20)	0.42
Vitamin supplement user, n (%)	33 (24)	61 (23)	0.65
Green tea intake (≥five cups per day), n (%)	36 (25)	71 (25)	0.42
Coffee intake (≥five cups per day), n (%)	4 (3)	5 (2)	0.83
Total energy intake (kcal/day), mean (SE)[b,c]	1,810 (12)	1,807 (8)	0.41
Fish and shellfish intake (g/day), mean (SE)[b,c]	109 (1)	110 (0.8)	0.75
Meat intake (g/day), mean (SE)[b,c]	68 (0.5)	68 (0.4)	0.15
Vegetable intake (g/day), mean (SE)[b,c]	297 (1)	296 (0.8)	0.20
Fruit intake (g/day), mean (SE)[b,c]	181 (2)	180 (1)	0.79
Isoflavone intake (mg/day), mean (SE)[b,c]	33 (0.7)	33 (0.5)	0.30

[a] P for Mantel–Haenszel test with matched-set 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

exclusion of subjects who provided a non-fasting blood sample, i.e., within 6 h after a meal, or who reported a history of chronic disease did not substantially change the results (data not shown). On the other hand, non-significant inverse associations were observed for plasma EGC and EC level when cases diagnosed before the first 4 years of follow-up were excluded, with adjusted ORs for the highest versus lowest group of 0.42 (95% CI 0.15–1.16; P for trend = 0.07) for EGC and 0.36 (95% CI 0.12–1.12; P for trend = 0.06) for EC.

Stratified analyses according to baseline menopausal status showed no remarkable difference between two strata regardless of tea polyphenol level (Table 3). In addition, no differential association was observed between subgroups defined by plasma genistein level, and dietary isoflavone and folate intake (data not shown).

Discussion

In this nested case–control study, we found no overall association between plasma tea polyphenols and the risk of breast cancer among Japanese women. Our findings are in general agreement with those of three prospective studies, which found no association between green tea intake and the risk of breast cancer [11–13], but contradict those of three case–control studies, which showed an inverse association [8–10]. Owing to a difference in exposure assessments between plasma tea polyphenols and green tea drinking habit, direct comparison of these results is difficult. Plasma tea polyphenol levels are partly determined by individual differences in absorption and metabolism, in addition to several conditions related to methods of drinking green tea, as mentioned in the Introduction. In fact, correlation coefficients between plasma levels and green tea intake were low, ranging between 0.16 for EC and 0.23 for EGC. For these reasons, plasma levels might better reflect interpersonal differences than green tea drinking habit. In this regard, our findings, based on such direct measurement, suggest that tea polyphenols at the levels measured in plasma might not play a major role in the etiology of breast cancer.

The observed absence of associations with breast cancer risk might be attributable to either or both poor

Table 2 Odds ratios (ORs) and 95% confidence intervals (CIs) of breast cancer according to plasma tea polyphenol levels

	Categories			*P* for trend
	Not detected	Under median value[a]	Over median value[a]	
EGC (ng/ml)				
Median[b]	Not detected	27.6	189.7	
No. of cases/no. of controls	97/202	28/43	19/43	
OR (95% CI)	1.00	1.40 (0.79–2.47)	0.90 (0.45–1.78)	0.87
Multivariate OR (95% CI)[c]	1.00	1.29 (0.65–2.54)	0.90 (0.42–1.96)	0.98
EC (ng/ml)				
Median[b]	Not detected	13.1	66.3	
No. of cases/no. of controls	109/221	19/33	16/34	
OR (95% CI)	1.00	1.20 (0.61–2.36)	0.97 (0.49–1.91)	0.96
Multivariate OR (95% CI)[c]	1.00	0.93 (0.43–2.02)	0.95 (0.43–2.08)	0.86
EGCG (ng/ml)				
Median[b]	Not detected	16.9	58.9	
No. of cases/no. of controls	113/233	16/27	15/28	
OR (95% CI)	1.00	1.25 (0.62–2.51)	1.12 (0.55–2.25)	0.61
Multivariate OR (95% CI)[c]	1.00	1.27 (0.58–2.80)	1.21 (0.52–2.80)	0.53
ECG (ng/ml)				
Median[b]	Not detected	7.1	14.7	
No. of cases/no. of controls	113/237	12/25	19/26	
OR (95% CI)	1.00	1.06 (0.49–2.29)	1.59 (0.82–3.08)	0.20
Multivariate OR (95% CI)[c]	1.00	1.28 (0.51–3.21)	1.75 (0.81–3.78)	0.15

EGC (-)-epigallocatechin, *EC* (-)-epicatechin, *EGCG* (-)-epigallocatechin-3-gallate, *ECG* (-)-epicatechin-3-gallate

[a] Among detected samples

[b] Median plasma level among control group for EGC, EC, EGCG, ECG

[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), alcohol intake (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 387 subjects with complete information of covariates

bioavailability or errors in the measurement of tea polyphenols. In fact, a large proportion of subjects had no detectable plasma level of tea polyphenols [17] despite a high and large variation in green tea intake: for example, only 15% of control women did not drink green tea, whereas 69% drank daily, of whom 36% drank more than five cups per day. However, we previously found an inverse association between plasma tea polyphenols, namely plasma ECG level, and the risk of gastric cancer among women in a nested case–control study within the JPHC study [18], which used the same population and method for measuring plasma tea polyphenols as the present study. This finding would argue against the possible explanation for the lack of association as being due to poor bioavailability and measurement error. Meanwhile, given that at least 20% of our subjects had detectable plasma levels of tea polyphenols despite their poor bioavailability, Japanese might represent a unique population in which to evaluate the effect of tea polyphenols on the risk of breast cancer. Our findings therefore suggest that tea polyphenols are unlikely to reduce the risk of breast cancer even at relatively high detectable concentrations achievable from habitual green tea drinking only.

One possible concern is that the long storage of blood samples might have lead to a large proportion of subjects having no detectable plasma levels of tea polyphenols due to degradation in plasma. No data for the effect of storage time on plasma level are available. In the present study, blood samples of cases and controls were stored under the same conditions. In addition, we matched for date of blood collection between cases and controls and measured plasma levels in the same batch to minimize measurement errors. Degradation of tea polyphenols in plasma is therefore unlikely to explain the observed absence of associations with breast cancer risk.

We found non-significant inverse associations between plasma EGC and EC level and the risk of breast cancer when cases diagnosed before the first 4 years of follow-up were excluded. The exclusion of these subjects might have minimized the possible influence of existing diseases and

Table 3 Odds ratios (ORs) and 95% confidence intervals (CIs) of breast cancer according to plasma tea polyphenol levels by baseline menopausal status

	Categories	
	Not detected	Detected
Premenopausal women		
EGC		
No. of cases/no. of controls	40/85	19/33
Multivariate OR (95% CI)[a]	1.00	1.44 (0.58–3.58)
EC		
No. of cases/no. of controls	46/91	13/27
Multivariate OR (95% CI)[a]	1.00	1.15 (0.43–3.11)
EGCG		
No. of cases/no. of controls	46/99	13/19
Multivariate OR (95% CI)[a]	1.00	1.78 (0.66–4.79)
ECG		
No. of cases/no. of controls	47/100	12/18
Multivariate OR (95% CI)[a]	1.00	1.67 (0.62–4.50)
Postmenopausal women		
EGC		
No. of cases/no. of controls	52/108	28/52
Multivariate OR (95% CI)[b]	1.00	0.95 (0.42–2.18)
EC		
No. of cases/no. of controls	58/120	22/40
Multivariate OR (95% CI)[b]	1.00	1.11 (0.43–2.84)
EGCG		
No. of cases/no. of controls	62/124	18/36
Multivariate OR (95% CI)[b]	1.00	1.22 (0.50–2.95)
ECG		
No. of cases/no. of controls	61/127	19/33
Multivariate OR (95% CI)[b]	1.00	1.91 (0.72–5.07)

EGC (-)-epigallocatechin, *EC* (-)-epicatechin, *EGCG* (-)-epigallocatechin-3-gallate, *ECG* (-)-epicatechin-3-gallate

[a] 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), alcohol intake (non-drinkers, occasional drinkers, 50 (g/week), 50–100 (g/week), 100+ (g/week) among regular drinkers (ethanol))

[b] 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 intake (non-drinkers, occasional drinkers, 50 (g/week), 50–100 (g/week), 100+ (g/week) among regular drinkers (ethanol))

their symptoms on green tea drinking habit, other lifestyle factors, and health behavior. Meanwhile, no association was observed when subjects who reported a history of chronic disease were excluded, indicating that the present findings were unlikely to have been affected by a history of chronic diseases. Moreover, it is unlikely that subjects changed their dietary habits, particularly green tea drinking habit, due to the presence of subclinical breast cancer. Given the relatively small number of cases in this study, these findings might rather be due to chance.

The association between green tea drinking and the risk of breast cancer may differ by menopausal status. A large population-based case–control study in Shanghai (n = 3454 cases), however, showed a lower risk of breast cancer among regular green tea drinkers than non-drinkers for both pre- and postmenopausal women [8]. Further, our study observed no association regardless of menopausal status. On the other hand, several previous studies have suggested effect modification of green tea intake by dietary factors and genetic polymorphisms [10, 13]. In a case–control study in Asian-Americans, a risk-reducing effect of green tea was primarily observed among subjects whose soy intake was low [10], while a nested case–control study in Singapore showed a protective effect of green tea against breast cancer among women with high-activity genotypes of the *methylenetetrahydrofolate reductase* (*MTHFR*) and *thymidylate synthase* (*TYMS*) genes [13]. This association was stronger among those whose dietary folate intake was low. Subsequent studies, however, have failed to replicate effect modification by soy and folate intake [8, 12]. Similarly, our stratified analyses suggested no differential association between subgroups defined by plasma genistein level, or dietary isoflavone and folate intake.

Our study has several methodological advantages over previous studies of green tea drinking and the risk of breast cancer. First, this is the first epidemiological study to directly measure tea polyphenols in plasma samples as an exposure assessment, these being the substances in green tea which putatively exert protective effects against breast cancer. Their direct measurement may allow us to elucidate the mechanisms by which green tea might influence breast cancer development. Second, 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. 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 also warrant mention. First, we measured tea polyphenols in plasma samples only once for each individual. Green tea drinking is a personal habit, and consumption levels of most individuals are assumed to be relatively stable over time in Japan, as suggested by our validation study [19, 20], which showed relatively high reproducibility of repeated measurements of green tea intake by FFQ (correlation coefficient = 0.64 for one-year interval and 0.54 for five-year interval) (unpublished data). By comparison, pharmacokinetic studies in humans have shown that the half-life of EGCG in blood ranges from 2 to 5 h [17], and that 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 therefore matched fasting time between cases and controls. 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 in 2002, 32.7 in Japan and 101.1 in United States for comparison) [21]. 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 a previous report showing the difficulty of extrapolating relative risk estimates for a sub-cohort to an entire cohort. This difficulty might in fact be inherent to prospective studies in general [22].

Allowing for these methodological issues, we found no overall association between plasma tea polyphenols and the risk of breast cancer in a nested case–control study in Japan. This finding suggests that tea polyphenols are unlikely to reduce the risk of breast cancer substantially, even at relatively high detectable concentrations.

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Conflict of interest statement All of the authors declare that they have no conflict of interest in connection with this paper.

Appendix

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Dietary Isoflavone Intake, Polymorphisms in the CYP17, CYP19, 17β-HSD1, and SHBG Genes, and Risk of Breast Cancer in Case-Control Studies in Japanese, Japanese Brazilians, and Non-Japanese Brazilians

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We tested the hypothesis that polymorphisms in cytochrome P450c17α (CYP17), aromatase (CYP19), 17β-hydroxysteroid dehydrogenase type I (17β-HSD1) and sex hormone-binding globulin (SHBG) genes may modify the association between isoflavone intake and breast cancer risk. We conducted hospital-based, case-control studies in Nagano, Japan and São Paulo, Brazil. A total of 846 pairs (388 Japanese, 79 Japanese Brazilians, and 379 non-Japanese Brazilians) completed validated food frequency questionnaires. Four single nucleotide polymorphisms (SNPs) in CYP17 (rs743572), CYP19 (rs10046), 17β-HSD1 (rs605059), and SHBG (rs6259) genes were genotyped. We found no association between the 4 SNPs and breast cancer risk. In combination analyses of isoflavone intake and SNPs, an inverse association between intake and risk was limited to women with at least one A allele of the rs605059 polymorphism for all 3 populations, albeit without statistical significance. For the rs6259 polymorphism, the inverse association was limited to postmenopausal Japanese with the GG genotype (odds ratio [OR] for highest vs. lowest tertile = 0.50, 95% confidence interval [CI] = 0.29–0.87; *P* for trend < 0.01), and to non-Japanese Brazilians with at least one A allele (OR for consumers vs. nonconsumer = 0.21, 95% CI = 0.06–0.77). We found no remarkable difference for the rs743572 and rs10046 polymorphisms. Our findings suggest that polymorphisms in the 17β-HSD1 and SHBG genes may modify the association between isoflavone intake and breast cancer risk.

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 high isoflavone intake to low breast cancer risk has been hypothesized (2). A recent meta-analysis supported this hypothesis and found a small decrease in breast cancer risk with higher soy intake (3). In contrast, a more recent meta-analysis indicated that the 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 owe to the similarity in the chemical structures of isoflavones and the human estrogen hormone, and the former's consequent binding affinity to estrogen receptors (6,7). Isoflavones can therefore act as estrogen agonists and antagonists competing for estradiol at the receptor complex (5). It has also been suggested that isoflavones may influence breast cancer risk by altering the biosynthesis, metabolism, and bioavailability of endogenous hormones. In this regard, isoflavones have been shown to inhibit aromatase (CYP19) (8–10) and 17β-hydroxysteroid dehydrogenase type I (17β-HSD1) (10–12) and to increase the synthesis of sex hormone-binding globulin (SHBG) (13,14). These findings in turn suggest that isoflavone might interact with these genes in the development of breast cancer.

Few studies have investigated whether genetic variants of genes involved in the biosynthesis, metabolism, and bioavailability of endogenous hormones modify the association between phytoestrogen exposure and risk of breast cancer (15,16). McCann et al. (15) reported that the risk-reducing effect of lignan intake on breast cancer was observed among premenopausal Caucasian women with at least one A2 allele of polymorphism in the cytochrome P450c17α (CYP17) gene but not among those with the A1A1 genotype (15). A similar result was found in a population-based case-control study in Germany in which an inverse association of plasma enterolactone and lignan intake with breast cancer risk was found among premenopausal women with the A2A2 genotype in the rs743572 polymorphism of the CYP17 gene (16). To our knowledge, however, the possible joint effect of phytoestrogen exposure and polymorphisms in the CYP19, 17β-HSD1, and SHBG genes on breast cancer risk has not been investigated.

Here, to test the hypothesis that polymorphisms in the CYP17, CYP19, 17β-HSD1, and SHBG genes might modify the association between isoflavone intake and breast cancer risk, we conducted hospital-based case-control studies in Nagano, Japan and São Paulo, Brazil, targeting 3 populations with substantially different intake of isoflavone and distribution of polymorphisms in the CYP17, CYP19, 17β-HSD1, and SHBG genes, namely Japanese living in Japan, Japanese Brazilians living in São Paulo and non-Japanese Brazilians living in São Paulo.

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 to 74 yr with newly diagnosed and histologically confirmed invasive breast cancer. Cases were recruited between 2001 and 2005 at 4 hospitals in Nagano and between 2001 and 2006 at 8 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 Nagano study, eligible controls were selected from medical checkup examinees in 2 of the 4 hospitals and confirmed not to have cancer. One control was matched for each case by age (within 3 yr) 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 case by age (within 5 yr) and ethnicity.

Among potential controls, 22 patients refused to participate (participation rate = 96%). Eventually, we obtained written informed consent from 472 matched pairs (83 Japanese Brazilians and 389 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.

Questionnaire

Participants in Nagano were asked to complete a self-administered questionnaire, whereas those in São Paulo were interviewed by trained interviewers using a structured questionnaire. 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 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 (17–19). 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 (20,21). 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 (22) and the United States Department of Agriculture (USDA) food composition tables for the Brazilian version (23). For some Japanese-specific foods in the Brazilian version, the Japanese Standard Tables of Food Composition 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 4 consecutive 7-day dietary records, one conducted in each the 4 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 (18). 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 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 (19).

Genotyping

Genomic DNA samples were extracted from the peripheral blood using Qiagen FlexiGene DNA Kits (Qiagen K.K., Tokyo, Japan) according to the manufacturer's protocol. We selected 4 single-nucleotide polymorphisms (SNPs) in CYP17 (rs743572), CYP19 (rs10046), 17β-HSD1 (rs605059), and SHBG (rs6259); these genes were the most frequently studied SNPs in relation to breast cancer risk (24–31). Genotyping of the 4 SNPs was performed by a commercial laboratory (Genetic Lab, Inc., Sapporo, Japan) using the TaqMan SNP Genotyping Assays developed by Applied Biosystems (Foster City, CA; Table 1). Cases and matched controls were analyzed in the same well by laboratory personnel who did not know the case-control status. As quality control assessment, we genotyped 6 SNPs of 4 genes (N-acetyltransferase 2 [NAT2], CYP17, CYP19, and cytochrome P450 2E1 [CYP2E1]) in our laboratory using about 24% of the samples in this study. The concordance rates between Genetic Lab. and our laboratory were varied between 97.6% and 99.5% among the 6 SNPs. In particular, the concordance rates of rs743572 and rs10046 polymorphism were 98.3% and 97.6%, respectively.

Statistical Analysis

We excluded subjects who reported extremely low or high total energy intake ($<$500 or $\geq$4,000 Kcal) or had no DNA sample, leaving 388 pairs of Japanese, 79 pairs of Japanese Brazilians, and 379 pairs of non-Japanese Brazilians for use in these analyses. 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 chi-square 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 nonconsumers 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 intakes and the SNP of interest. The following variables, selected mainly based on the basis of 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 yr, 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 2-sided, and significance level was set at $P < 0.05$. All statistical analyses

TABLE 1
Single-nucleotide polymorphisms (SNPs) in CYP17, CYP19, 17β-HSD1, and SHBG genes and their allele frequency[a]

Gene	SNP rs Number	Amino Acid Change	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
CYP17A1	rs743572	5′-UTR	T/C	0.45	0.50	0.39
CYP19A1	rs10046	3′-UTR	C/T	0.43	0.44	0.42
HSD17B1	rs605059	Ser312Gly	G/A	0.47	0.49	0.48
SHBG	rs6259	Asp327Asn	G/A	0.12	0.17	0.10

[a]Abbreviations are as follows: CYP, cytochrome P450; 17β-HSD1, 17β-hydroxysteroid dehydrogenase type I; SHBG, sex hormone-binding globulin.

were performed with SAS software version 9.1 (SAS Institute, Inc., Cary, NC).

RESULTS

Characteristics of cases and controls were described in a previous report (32) (data not shown in table). 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, whereas 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 46.2 for Japanese, 23.5 for Japanese Brazilians, and 4.4 for non-Japanese Brazilians.

The distribution of SNPs in the CYP17 (rs743572), CYP19 (rs10046), 17β-HSD1 (rs605059), and SHBG (rs6259) genes is presented in Tables 1 and 2. Among controls in each population, genotype frequencies of each SNP were consistent with the Hardy Weinberg equilibrium except for the rs743572 polymorphism in non-Japanese Brazilians ($P = 0.04$). The prevalence of the minor allele in the rs743572 and rs6259 polymorphisms was somewhat higher in the control group of Japanese and Japanese Brazilians than in that of non-Japanese Brazilians. None of the individual SNPs was associated with the risk of breast cancer for any of the 3 populations (Table 2). In stratified analyses by menopausal status, none of the adjusted ORs showed statistical significance for all 4 SNPs in any of the 3 populations except for ORs for premenopausal women with the CC vs. TT genotype of the rs743572 polymorphism (OR = 2.88, 95% CI = 1.30–6.37) and for postmenopausal women with the CT vs. CC genotype of the rs10046 polymorphism (OR = 0.61, 95% CI = 0.40–0.95) among non-Japanese Brazilians (data not shown).

In a previous report, we found a nonsignificant inverse association between isoflavone intake and the risk of breast cancer in postmenopausal Japanese but a statistically significant inverse association for Japanese Brazilians and non-Japanese Brazilians (32). Analyses of combinations of isoflavone intake and the rs605059 polymorphism in the 17β-HSD1 gene revealed that the risk of breast cancer only decreased with increasing isoflavone intake for women with at least one A allele for postmenopausal Japanese (OR for highest vs. lowest tertile = 0.62, 95% CI = 0.28–1.39; P for trend = 0.03), Japanese Brazilians (OR for highest vs. lowest median = 0.74, 95% CI = 0.28–2.00), and non-Japanese Brazilians (OR for consumers vs. nonconsumers = 0.51, 95% CI = 0.28–0.94), although no statistically significant interaction was found (P for interaction = 0.49, 0.15, and 0.33, respectively; Tables 3 and 4). For the rs6259 polymorphism in the SHBG gene, the significant inverse association was limited to women with the GG genotype for postmenopausal Japanese (OR for highest vs. lowest tertile = 0.50, 95% CI = 0.29–0.87; P for trend < 0.01) and Japanese Brazilians (OR for highest vs. lowest median = 0.38, 95% CI = 0.16–0.89; P for interaction = 0.06 and 0.32, respectively). In contrast, the association was limited to women with at least one A allele for non-Japanese Brazilians (OR for consumers vs. nonconsumer = 0.21, 95% CI = 0.06–0.77; P for interaction = 0.16). We found no remarkable difference in the association between isoflavone intake and breast cancer risk by polymorphisms in the CYP17 and CYP19 genes.

DISCUSSION

In these case-control studies of Japanese, Japanese Brazilians, and non-Japanese Brazilians, we found that an inverse association between isoflavone intake and breast cancer risk only appeared among women with at least one A allele of the rs605059 polymorphism in the 17β-HSD1 gene. Moreover, an inverse association was limited to women with the GG

TABLE 2

Odds ratios (ORs) and 95% confidence intervals (CIs) of breast cancer according to polymorphisms in CYP17, CYP19, 17β-HSD1, and SHBG genes[a]

	Japanese Living in Nagano, Japan				Japanese Brazilians Living in São Paulo, Brazil				Non-Japanese Brazilians Living in São Paulo, Brazil			
	No.				No.				No.			
	Case	Control	OR[a]	95% CI	Case	Control	OR[a]	95% CI	Case	Control	OR[b]	95% CI
CYP17A1 gene (rs743572)												
TT	111	122	1.00		17	23	1.00		135	130	1.00	
TC	189	182	1.30	(0.91–1.86)	48	33	2.34	(0.93–5.88)	185	200	0.94	(0.69–1.29)
CC	88	84	1.42	(0.92–2.18)	13	23	0.53	(0.17–1.64)	59	49	1.08	(0.68–1.71)
TC + CC	277	266	1.33	(0.95–1.87)	61	56	1.53	(0.68–3.45)	244	249	0.97	(0.71–1.31)
CYP19A1 gene (rs10046)												
CC	118	125	1.00		24	22	1.00		133	121	1.00	
CT	188	194	1.05	(0.73–1.51)	41	44	0.97	(0.43–2.16)	179	200	0.82	(0.59–1.13)
TT	82	69	1.30	(0.82–2.05)	14	13	1.02	(0.39–2.72)	67	58	1.01	(0.65–1.57)
CT + TT	270	263	1.12	(0.80–1.57)	55	57	0.99	(0.47–2.09)	246	258	0.86	(0.63–1.17)
HSD17B1 gene (rs605059)												
GG	108	109	1.00		21	18	1.00		103	101	1.00	
GA	199	187	1.04	(0.71–1.53)	36	45	0.84	(0.37–1.95)	187	187	0.98	(0.70–1.39)
AA	78	88	0.87	(0.54–1.38)	13	16	1.19	(0.39–3.65)	84	88	0.94	(0.62–1.43)
GA + AA	277	275	0.99	(0.68–1.43)	49	61	0.93	(0.43–2.00)	271	275	0.97	(0.70–1.34)
SHBG gene (rs6259)												
GG	304	303	1.00		62	55	1.00		317	306	1.00	
GA	80	78	0.89	(0.60–1.33)	17	22	0.59	(0.25–1.39)	57	71	0.74	(0.50–1.09)
AA	4	7	0.28	(0.06–1.30)	0	2	—	—	5	1	5.77	(0.64–51.71)
GA + AA	84	85	0.83	(0.56–1.22)	17	24	0.53	(0.23–1.22)	62	72	0.80	(0.54–1.17)

[a]Abbreviations are as follows: CYP, cytochrome P450, 17β-HSD1, 17β-hydroxysteroid dehydrogenase type I; SHBG, sex hormone-binding globulin.

[b]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 yr (no, less than 3 days/mo, 1–4 days/wk, more than 5 days/wk), and vitamin supplement use (yes, no).

TABLE 3

Odds ratios (ORs) and 95% confidence intervals (CIs) of breast cancer for combinations of dietary intake of isoflavone and polymorphisms in CYP17, CYP19, 17β-HSD1, and SHBG genes among Japanese[a]

	All Subjects				Premenopausal Women				Postmenopausal Women			
	Isoflavone Intake (mg/Day), Tertile Category				Isoflavone Intake (mg/Day), Tertile Category				Isoflavone Intake (mg/Day), Tertile Category			
	1	2	3	*P* for Trend	1	2	3	*P* for Trend	1	2	3	*P* for Trend
CYP17A1 gene (rs743572)												
TT												
No.[b]	51/40	29/39	31/43		28/19	13/16	10/11		23/31	16/23	21/32	
OR[c]	1.00	0.76	0.67	0.22	1.00	0.57	0.59	0.62	1.00	0.79	0.54	0.20
(95% CI)		(0.37–1.57)	(0.32–1.43)			(0.21–1.53)	(0.19–1.84)			(0.32–1.98)	(0.23–1.30)	
TC + CC												
No.[b]	100/89	89/90	88/87		51/47	39/27	35/15		49/42	50/63	53/72	
OR[c]	1.14	1.06	1.03	0.76	0.85	1.12	1.55	0.21	0.98	0.78	0.65	0.19
(95% CI)	(0.64–2.03)	(0.61–1.86)	(0.56–1.91)		(0.40–1.81)	(0.50–2.51)	(0.63–3.79)		(0.45–2.10)	(0.37–1.64)	(0.31–1.36)	
	P for interaction = 0.78				*P* for interaction = 0.18				*P* for interaction = 0.91			
CYP19A1 gene (rs10046)												
CC												
No.[b]	46/36	36/46	36/43		28/19	15/15	18/11		18/17	21/31	18/32	
OR[c]	1.00	0.62	0.53	0.14	1.00	0.68	0.88	0.67	1.00	0.77	0.49	0.09
(95% CI)		(0.30–1.26)	(0.25–1.14)			(0.26–1.80)	(0.32–2.45)			(0.31–1.90)	(0.19–1.27)	
CT + TT												
No.[b]	105/93	82/83	83/87		51/47	37/28	27/15		54/46	45/55	56/72	
OR[c]	0.78	0.79	0.75	0.72	0.66	0.81	1.05	0.24	1.10	0.90	0.75	0.16
(95% CI)	(0.43–1.42)	(0.43–1.45)	(0.39–1.43)		(0.31–1.39)	(0.36–1.82)	(0.42–2.64)		(0.49–2.46)	(0.40–2.03)	(0.34–1.66)	
	P for interaction = 0.35				*P* for interaction = 0.52				*P* for interaction = 0.81			
HSD17B1 gene (rs605059)												
GG												
No.[b]	39/40	28/36	41/33		20/23	14/19	17/7		19/17	14/17	24/26	
OR[c]	1.00	0.96	1.51	0.31	1.00	0.80	2.76	0.13	1.00	1.02	1.02	0.82
(95% CI)		(0.44–2.11)	(0.69–3.30)			(0.31–2.11)	(0.89–8.58)			(0.36–2.91)	(0.40–2.57)	
GA + AA												
No.[b]	111/89	88/90	78/96		59/43	38/24	28/18		52/46	50/66	50/78	
OR[c]	1.31	1.13	0.80	0.08	1.50	1.76	1.44	0.92	1.19	0.89	0.62	**0.03**[d]
(95% CI)	(0.71–2.41)	(0.62–2.06)	(0.42–1.51)		(0.70–3.19)	(0.77–4.01)	(0.58–3.59)		(0.52–2.70)	(0.40–1.98)	(0.28–1.39)	
	P for interaction = 0.12				*P* for interaction = 0.14				*P* for interaction = 0.49			
SHBG gene (rs6259)												
GG												
No.[b]	123/104	90/103	91/96		57/55	38/32	36/17		66/49	52/71	55/79	
OR[c]	1.00	0.91	0.81	0.30	1.00	1.13	1.72	0.12	1.00	0.64	**0.50**	**<0.01**
(95% CI)		(0.59–1.39)	(0.51–1.30)			(0.61–2.12)	(0.82–3.61)			(0.37–1.11)	**(0.29–0.87)**	
GA + AA												
No.[b]	28/25	28/26	28/34		22/11	14/11	9/9		6/14	14/15	19/25	
OR[c]	0.81	0.71	0.77	0.94	1.75	1.17	0.92	0.15	**0.28**	0.79	0.59	0.24
(95% CI)	(0.41–1.62)	(0.38–1.35)	(0.40–1.48)		(0.74–4.15)	(0.46–2.95)	(0.32–2.68)		**(0.10–0.85)**	(0.33–1.87)	(0.28–1.25)	
	P for interaction = 0.92				*P* for interaction = 0.26				*P* for interaction = 0.06			

[a]Abbreviations are as follows: CYP, cytochrome P450, 17β-HSD1, 17β-hydroxysteroid dehydrogenase type 1; SHBG, sex hormone-binding globulin.

[b]No. of cases/No. of controls.

[c]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 yr (no, less than 3 days/mo, 1–4 days/wk, more than 5 days/wk), 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 smokers), moderate physical activity in the past 5 yr (no, less than 3 days/mo, 1–4 days/wk, more than 5 days/wk), and vitamin supplement use (yes, no).

[d]ORs and 95% CIs with statistical significance are written in bold.

TABLE 4

Odds ratios (ORs) and 95% confidence intervals (CIs) of breast cancer for combinations of dietary intake of isoflavone and polymorphisms in CYP17, CYP19, 17β-HSD1, and SHBG genes among Japanese Brazilians and non-Japanese Brazilians[a]

	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	Nonconsumers	Consumers
CYP17A1 gene (rs743572)				
TT				
No.[b]	11/12	6/11	121/110	14/20
OR[c]	1.00	0.47	1.00	0.73
(95% CI)		(0.10–2.09)		(0.33–1.60)
TC + CC				
No.[b]	34/27	27/29	222/208	22/41
OR[c]	1.93	0.86	1.00	**0.49**[d]
(95% CI)	(0.65–5.72)	(0.31–2.38)	(0.73–1.38)	**(0.27–0.91)**
	P for interaction = 0.96		*P* for interaction = 0.43	
CYP19A1 gene (rs10046)				
CC				
No.[b]	15/10	9/12	120/104	13/17
OR[c]	1.00	0.46	1.00	0.63
(95% CI)		(0.13–1.58)		(0.27–1.44)
CT + TT				
No.[b]	31/29	24/28	223/214	23/44
OR[c]	0.89	0.48	0.88	**0.46**
(95% CI)	(0.33–2.41)	(0.16–1.42)	(0.62–1.23)	**(0.25–0.84)**
	P for interaction = 0.83		*P* for interaction = 0.73	
HSD17B1 gene (rs605059)				
GG				
No.[b]	13/12	8/6	91/86	12/15
OR[c]	1.00	1.78	1.00	0.81
(95% CI)		(0.32–10.07)		(0.36–1.86)
GA + AA				
No.[b]	27/27	22/34	247/230	24/45
OR[c]	1.93	0.74	1.04	**0.51**
(95% CI)	(0.61–6.14)	(0.28–2.00)	(0.73–1.47)	**(0.28–0.94)**
	P for interaction = 0.15		*P* for interaction = 0.33	
SHBG gene (rs6259)				
GG				
No.[b]	38/27	24/28	285/258	32/48
OR[c]	1.00	**0.38**	1.00	0.64
(95% CI)		**(0.16–0.89)**		(0.38–1.06)
GA + AA				
No.[b]	8/12	9/12	58/59	4/13
OR[c]	0.29	0.29	0.88	0.21
(95% CI)	(0.08–1.04)	(0.07–1.21)	(0.58–1.34)	(0.06–0.77)
	P for interaction = 0.32		*P* for interaction = 0.16	

[a]Abbreviations are as follows: CYP, cytochrome P450, 17β-HSD1, 17β-hydroxysteroid dehydrogenase type I; SHBG, sex hormone-binding globulin.

[b]No. of cases/No. of controls.

[c]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 yr (no, less than 3 days/mo, 1–4 days/wk, more than 5 days/wk), and vitamin supplement use (yes, no).

[d]ORs and 95% CIs with statistical significance are written in bold.

genotype of the rs6259 polymorphism in the SHBG gene for postmenopausal Japanese and Japanese Brazilians and to women with at least one A allele for non-Japanese Brazilians. Our findings support the hypothesis that polymorphisms in genes related to the biosynthesis, metabolism, and bioavailability of endogenous hormones may modify the association between isoflavone intake and breast cancer risk.

The rs605059 polymorphism of the 17β-HSD1 gene results in an amino acid change from serine (A allele) to glycine (G allele) at position 312 but does not affect the catalytic or immunological properties of the enzyme (24). Previous studies have found no overall association between the rs605059 polymorphism and risk of breast cancer, which is in general agreement with our findings (24–26). Although interactions between phytoestrogen exposure and polymorphisms in the 17β-HSD1 gene in the risk of breast cancer has not been investigated, Dai et al.'s (33) population-based case-control study in Shanghai reported a significant interaction between isoflavone intake and the rs605059 polymorphism in the risk of endometrial cancer in which an inverse association was only seen among premenopausal women with at least one A allele. In this study, the risk of breast cancer decreased with increasing isoflavone intake only for women with at least one A allele of the rs605059 polymorphism in all 3 populations. Although the interactions were not statistically significant, the overall consistency of findings in the 3 populations suggests that isoflavones may reduce the risk of breast cancer via a mechanism involving the 17β-HSD1 gene.

We found that the risk of breast cancer decreased with increasing isoflavone intake only for women with at least one A allele of the rs605059 polymorphism, even though this polymorphism does not change the catalytic properties of the enzyme. Although the mechanism underlying this observation remains unclear, one possibility comes from Dai et al. (33) who hypothesized from their findings that the amino-acid alteration from serine to glycine may produce a structural change in the 17β-HSD1 binding domain, which in turn results in a substantial loss in enzyme affinity with isoflavones.

The rs6259 polymorphism of the SHBG gene results in an amino acid change substitution of asparagine (G allele) to aspartic acid (A allele) at position 327. The A allele is thought to create SHBG molecules with reduced clearance, which results in higher circulating SHBG levels (34). It has therefore been hypothesized that the A allele is associated with a decreased risk of breast cancer. Overall, however, the few studies that have been reported to date do not support this hypothesis, which is consistent with our findings (27,28). In their cross-sectional study of 1988 healthy postmenopausal women from the European Prospective Investigation of Cancer and Nutrition-Norfolk cohort, moreover, Low et al. (34) reported that plasma SHBG levels were positively associated with urinary isoflavones in women carrying at least one A allele but not in women carrying the GG genotype. This implies that the decrease in breast cancer risk associated with isoflavone exposure might be more prominent among women with at least one A allele. We found that the inverse association between isoflavone intake and breast cancer risk was limited to women with at least one A allele for non-Japanese Brazilians, which supports the hypothesis, but was limited to women with the GG genotype for postmenopausal Japanese and Japanese Brazilians. This inconsistency in findings might reflect the amount of intake on the basis that the results were in fact consistent among populations with low intake. Nevertheless, the reason for the inconsistency remains unclear, and both findings might merely be due to chance. If at least one finding is not due to chance, however, our findings suggest that isoflavones may reduce the risk of breast cancer via a mechanism that involves the SHBG gene.

We failed to replicate previous studies, which have shown an interaction between lignan exposure and the rs743572 polymorphism in the risk of premenopausal breast cancer (15,16). In this study, we found no association between isoflavone intake and breast cancer risk among premenopausal Japanese women. Because of the small number of cases for both Japanese Brazilians and non-Japanese Brazilians, we did not perform stratified analyses according to menopausal status, which might account for the inconsistency. In addition, we found no remarkable difference in the association between isoflavone intake and breast cancer risk by rs10046 polymorphism. Because we evaluated only one SNP in the CYP19 gene, further studies based on a comprehensive evaluation of the gene would clarify the gene-nutrient interaction.

Our study has several methodological advantages over previous studies. First, and unique to this study, we assessed gene-nutrient interactions using 3 populations with different isoflavone intake. For example, isoflavone intake differed considerably among the 3 populations, with median levels (interquartile rage) in the control group (mg/day) of 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. Second, we analyzed data from 3 populations, meaning that the generalizability of those results that were consistent among them is greater than would be possible for a single population.

Several limitations of the study also 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 those of the general population due to health consciousness or disease, might have lead to selection bias. Third, because the evaluation of gene-nutrient interactions was performed in a relatively small number of cases, power to evaluate interactions between isoflavone intake and genotype was limited. This may have also limited the interpretability of the results.

Allowing for these methodological issues, our findings support the hypothesis that polymorphisms in the 17β-HSD1 and

SHBG genes may modify the association between isoflavone intake and breast cancer risk. Further, they provide additional evidence that the mechanisms by which isoflavone may reduce the risk of breast cancer might be mediated via alteration of the biosynthesis, metabolism, and bioavailability of endogenous hormones.

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Chemopreventive effects of 13α,14α-epoxy-3β-methoxyserratan-21β-ol (PJJ-34), a serratane-type triterpenoid, in a rat multi-organ carcinogenesis bioassay

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ABSTRACT

A novel serratane-type triterpenoid, 13α,14α-epoxy-3β-methoxyserratan-21β-ol (PJJ-34) derived from cuticles of *Picea jezoensis* Carr. var. *jezoensis*, has proved to be highly effective at suppressing carcinogenesis both in vitro and in vivo. To investigate possible anti-carcinogenic efficacy at the whole-body level, male Fischer 344 rats were subjected to an established rat multi-organ carcinogenesis bioassay (DMBDD model). After initiation with five carcinogens, groups 1–3 (20 in each) were intragastrically (i.g.) administered PJJ-34 dissolved in 1 ml of 0.5% CMC (5 times/week) at doses of 0, 5 and 10 mg/kg body weight (b.w.), respectively, until the end of week 30. PJJ-34 did not show apparent toxicity. Incidences of adenomas (100 → 75%) and carcinomas (63 → 30%) in the lung were significantly decreased in the 5 mg/kg b.w. group, and multiplicity of alveolar hyperplasias and total lung tumors (adenomas + carcinomas) were significantly reduced by both 5 and 10 mg/kg. The incidence of colorectal tumors was also significantly decreased in the 10 mg/kg group (63 → 28%) along with the multiplicity. Rat liver pre-neoplastic lesions, glutathione *S*-transferase placental form (GST-P) foci, and tumor development in the other organs were not affected. Immunohistochemical indices for proliferating cell nuclear antigen (PCNA) and cyclin D1 in normal alveolar epithelium of the lung were significantly suppressed at both doses. In conclusion, PJJ-34 is chemopreventive against lung and colon carcinogenesis without exerting apparent toxicity, and suppression of cell proliferation could play a key role in the underlying mechanisms.

1. Introduction

More than 24,000 chemicals are registered for use in Japan and humans are actually exposed to thousands of environmental chemicals with great diversity of pharmacological function and structure, simultaneously and/or sequentially during manufacture, distribution, use and disposal, or when they become pollutants in the air, water, or soil [1]. The accepted major factors for human carcinogenesis are smoking, infection, inflammation, poor nutrition

Abbreviations: BBN, *N*-butyl-*N*-(4-hydroxy-butyl)nitrosamine; DEN, diethylnitrosamine; DHPN, 2,2′-dihydroxy-di-*N*-propylnitrosamine; DMH, 1,2-dimethylhydrazine dihydrochloride; GST-P, glutathione *S*-transferase placental form; MNU, *N*-methyl-*N*-nitrosourea; PCNA, proliferating cell nuclear antigen; PJJ-34, 13α,14α-epoxy-3β-methoxy-serratan-21β-ol.

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and dietary carcinogens [2]. It is impossible to avoid all the risk factors in our environment and since cancer is now the leading cause of death in most countries, primary cancer prevention has naturally attracted attention. It has been argued that at least 50% of all cancers could be avoided by applying the existing etiologic knowledge [3]. Chemoprevention is one approach to reducing the burden of cancer and is recognized as both a clinical and basic science which is developing with incorporation of new in vitro and in vivo assays [4].

13α,14α-Epoxy-3β-methoxyserratan-21β-ol (PJJ-34), a newly-identified naturally-occurring serratane-type triterpenoid, is a promising chemopreventive agent which has proved to be highly effective for suppression of carcinogenesis both in vitro and in vivo [5]. More than ten years of our efforts to discover chemopreventive agents have been focused on serratane-type triterpenoids extracted from the highly-developed cuticles of *Picea jezoensis* (Sieb. et Zucc.) Carr. var. *jezoensis* (Pinaceae; Japanese name: Ezomatsu) and from the stem bark of *P. jezoensis* (Sieb. et Zucc.) Carr. var. *hondoensis* (Mayr.) Rehder. Earlier papers documented detailed procedures for purification of these compounds and determination of chemical structures [6–8], including isolation of PJJ-34 [9]. Methodology for discovery of chemopreventive effects of these serratane-type triterpenoids was routinely consisted of in vitro and in vivo carcinogenesis assays: first, test compounds were studied for their inhibitory effects (Trypan-Blue assay) on Epstein-Barr virus early antigen (EBV-EA) activation induced by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) in Raji cells (EBV genome-carrying lymphoblastoid cells derived from a Burkitts lymphoma), and those showing more potent inhibitory potential than oleanolic acid (a well-established chemopreventive triterpenoid) were further evaluated for anti-tumorigenic effects on a two-stage mouse skin carcinogenesis model featuring 7,12-dimethylbenz[a]anthracene (DMBA) as an initiator and TPA as a promoter [5,10–14].

The medium-term rat multi-organ carcinogenesis bioassay system, generally known as the DMBDD model [15,16], features partly concomitant or partly sequential application of five genotoxic carcinogens, diethylnitrosamine (DEN), *N*-methyl-*N*-nitrosourea (MNU), *N*-butyl-*N*-(4-hydroxy-butyl)nitrosamine (BBN), 2,2′-dihydroxy-di-*N*-propylnitrosamine (DHPN), and 1,2-dimethylhydrazine dihydrochloride (DMH), with further modification of the model to obtain good tumor yields within 30 weeks [17]. Each carcinogen has different organ specificity and the DMBDD model allows induction of a variety of pre-neoplastic and neoplastic lesions at the whole-body level. The main targeted organs are the lung, colon, bladder, kidney, thyroid and liver. From accumulated experimental evidence, the DMBDD bioassay is very useful for investigation of the carcinogenic modifying potentials of various chemicals in the post-initiation phase, using neoplastic and/or established pre-neoplastic lesions as the endpoints [18].

In the present study, modifying effects of PJJ-34 were evaluated for the first time in the DMBDD model, the results providing some novel evidence in vivo.

2. Materials and methods

2.1. Animals, diet and chemical carcinogens

Male Fischer 344 rats were obtained at 5 weeks of age (Charles River Japan, Atsugi, Japan), housed four in plastic cages, and given CE-2 common basal diet (Clea Japan, Tokyo, Japan) and water ad libitum. They were kept in an environmentally controlled room maintained at a temperature of 22 ± 2 °C and a relative humidity of 50 ± 5%, with a 12-h light/dark cycle. Body weights, food consumption and water intake were measured weekly during the experimental period. After a 1-week acclimation period, the animals were used in this study. DEN, BBN and DMH were obtained from Tokyo Kasei Kogyo (Tokyo, Japan). MNU was from Wako Pure Chemical Industries (Osaka, Japan). DHPN was from Nacalai Tesque (Kyoto, Japan). DEN, MNU and DMH were dissolved in 0.9% physiological saline and used for intraperitoneal (i.p.) or subcutaneous (s.c.) injections. BBN and DHPN were administered to rats in their drinking water.

2.2. Extraction and synthesis of PJJ-34 from 3β-methoxyserrat-14-en-21β-ol (PJJ-1)

PJJ-34 (molecular formula: $C_{31}H_{52}O_3$) was isolated from the air-dried chopped cuticle of one *P. jezoensis* Carr. var. *jezoensis* tree (estimated over 300 years old) which growing at 1000 m in mountains around Hidaka town, Hokkaido, Japan, and the procedure with chemical structures was well documented [5,9]. The most abundant triterpene, 3β-methoxyserrat-14-en-21β-ol (PJJ-1) (1) was first obtained, and synthetic PJJ-34 was used in this study (Fig. 1a). In brief, PJJ-1 (30 g) was acetylated (Ac_2O/pyridine 1:1, 100 ml) to give 3β-methoxyserrat-14-en-21β-yl acetate (PJJ-1 acetate: 27.6 g) (2). A mixture of glacial AcOH (240 ml) and *c*-H_2SO4 (90 ml) was gradually added into (2) and was kept at room temperature for 24 h. Then, the mixture was poured into ice water and the resulting precipitate was extracted with $CHCl_3$. The extract was neutralized with 5% NaOH, washed with H_2O and dried over Na_2SO_4. Evaporation of $CHCl_3$ yielded a crystalline mass (26.7 g), which was subjected to SiO_2 column chromatography to afford 3β-methoxyserrat-13-en-21β-yl acetate (PJJ-1–13-en Ac: 20.1 g) (3). Treatment of compound (3) in boiling 0.3 N KOH/MeOH (600 ml) for 8 h and subsequent workup as usual furnished 3β-methoxyserrat-13-en-21β-ol (PJJ-1-13-en: 18.3 g) (4), followed by adding a solution of *m*-chloroperbenzoic acid (*m*-CPBA) in dry $CHCl_3$. After 24 h, the reaction mixture was washed and evaporated under reduced pressure, then the residue was purified by MPLC (240–400 mesh SiO_2) eluting with *n*-hexane-AcOEt (10: 1) to afford 13α,14α-epoxy-3β-methoxyserratan-21β-ol (PJJ-34: 15.8 g) (5) and 13β,14β-epoxy-3β-methoxyserratan-21β-ol (PJJ-43: 0.9 g) (6). Synthetic PJJ-34 (99% in purity) was identified by direct comparison with authentic natural PJJ-34, which was stored at 4 °C until use.

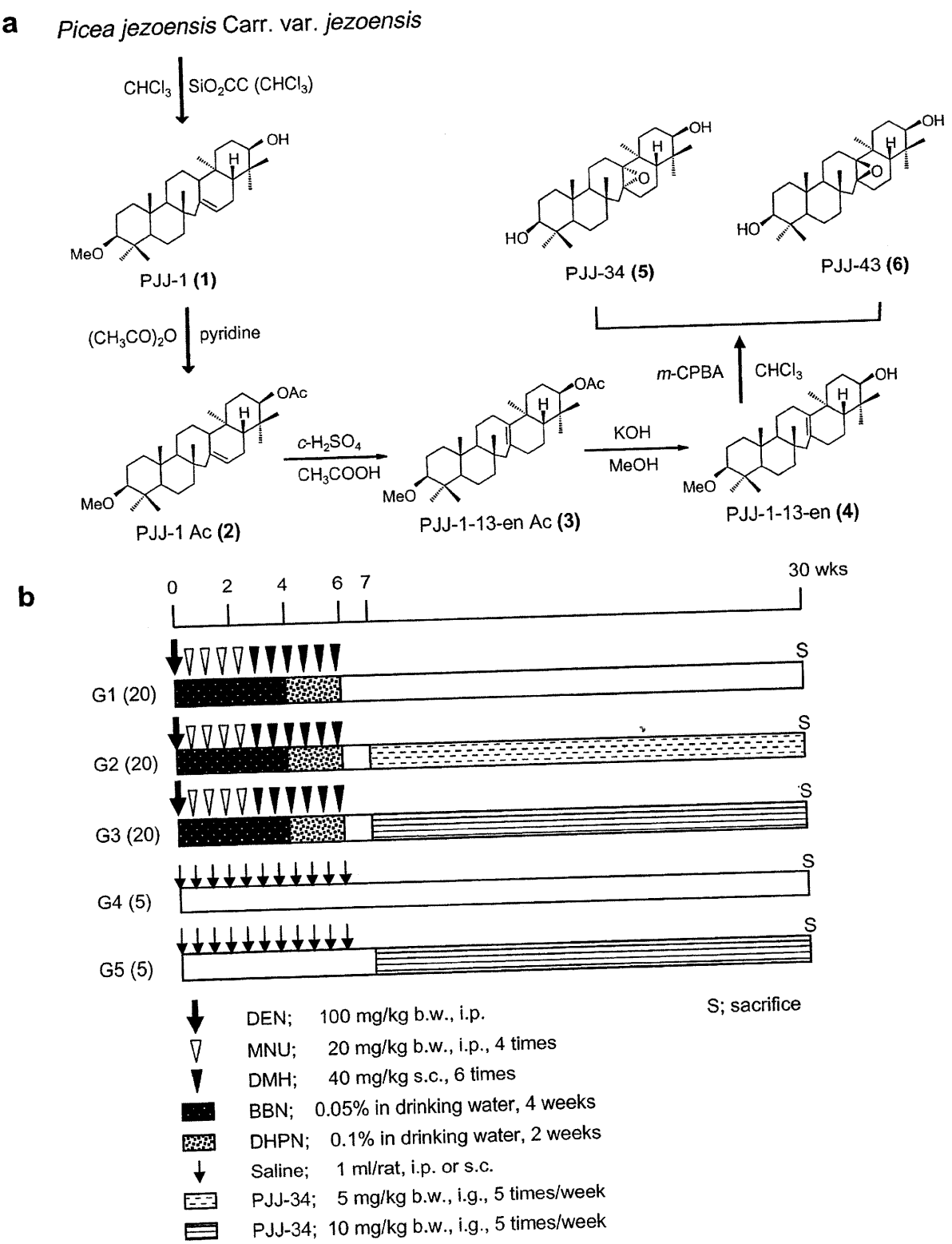

Fig. 1. Extraction and biosynthesis of PJJ-34, and experimental protocol of the DMBDD model used in this study. (a) PJJ-34 and analogues were extracted from the cuticles of a *Picea jezoensis* Carr. var. *jezoensis* tree [Ref. 5,9], which can also be synthesized from PJJ-1 and synthetic PJJ-34 (purity >99%).was used in this study. Compound (1) 3β-methoxyserrat-14-en-21β-ol; (2) 3β-methoxyserrat-14-en-21β-yl acetate; (3) 3β-methoxyserrat-13-en-21β-yl acetate; (4) 3β-methoxyserrat-13-en-21β-ol; (5) 13α,14α-epoxy-3β-methoxyserratan-21β-ol; and (6) 13β,14β-epoxy-3β-methoxyserratan-21β-ol. (b) The established rat multi-organ carcinogenesis bioassay (DMBDD model) was carried out with a modification as well as shown in a previous report [17].

2.3. Preliminary determination of the experimental doses of PJJ-34

To determine the experimental doses, 6-week-old, male F344 rats were preliminarily tested. Each time of dosing, PJJ-34 was dissolved in 0.5% CMC-Na (carboxymethyl cellulose sodium salt; Wako Pure Chemical Industries) at doses of 0, 1, 5, 10, and 20 mg/kg body weight (b.w.) for intragastric (i.g.) administration to animals 5 times per week for 4 weeks. As a result, some toxicological findings

were observed in the 20 mg/kg b.w. group, while 1, 5 and 10 mg/kg b.w. groups did not show any apparent toxicity (data not shown). Therefore, we decided to use 5 and 10 mg/kg b.w. as appropriate experimental doses in the present case.

2.4. Experimental protocol

The experimental design, which was approved by the Institutional Animal Care and Use Committee of Osaka City University Medical School, is shown in Fig. 1b. A total of 70, 6-week-old, male F344 rats were divided into five groups. Animals in groups 1–3 (20 rats in each) underwent the modified DMBDD regimen [17], consisting of a single injection of DEN (100 mg/kg b.w., i.p.) at the commencement of experiment, followed by MNU (20 mg/kg b.w., i.p.) for 4 times and DMH (40 mg/kg b.w., s.c.) for 6 times. At the same period, 0.05% BBN in drinking water was administered during experimental weeks 1–4, followed by 0.1% DHPN during weeks 5 and 6. Animals in groups 4 and 5 (5 rats in each) received the vehicle saline instead of the carcinogen injections. After the initiation period, all rats were maintained without any treatment for 1 week for recovery and for distinction of the post-initiation period from the initiation term. Then rats in DMBDD-treated groups 1–3 received i.g. administration of PJJ-34 dissolved in 0.5% CMC at doses of 0, 5 and 10 mg/kg b.w., respectively, 5 times per week until the end of the experiment. Animals in the non-DMBDD groups 4 and 5 were given PJJ-34 in 0.5% CMC at doses of 0 or 10 mg/kg b.w. (5 times/week, i.g.), respectively, and the experiment was terminated at the end of week 30.

2.5. Organ and tissue processing

At the time of sacrifice, rats were killed under ether anesthesia and any macroscopical abnormalities were recorded. The major organs were excised and the liver, kidneys and spleen were immediately weighed. The alimentary tract from the esophagus to the rectum, the urinary bladder, and lungs with the trachea and thyroid gland were inflated gently with 10% phosphate-buffered formalin solution. The colon was cut open along the longitudinal axis and extended flat between two sheets of papers. Several skin tumors as well as other visible lesions were also sampled. All these organs and tissues were fixed in the same formalin solution.

Approximately 3 mm-thick slices were prepared for histological examination from all organs taken. Single liver slices were made from the left, intermediate and caudate lobes. Lungs were divided into 5 different portions (the left lobe and 4 right sublobes) and slices were taken from each. Three slices were routinely cut from the colon with rectum as well as visible tumors. The Bladders were routinely cut into 8 slices. All of the formalin-fixed, paraffin-embedded tissues were routinely prepared for 3 μm-thick sections and stained with hematoxylin and eosin (HE). Incidence (%) and/or multiplicity data for neoplastic and pre-neoplastic lesions in the major target organs were evaluated in this study.

2.6. Immunohistochemistry for PCNA and Cyclin D1

In order to evaluate cell proliferation, sections from the lung and colon ($n = 18–20$ in groups 1–3) were immunohistochemically stained with proliferating cell nuclear antigen (PCNA), and cyclin D1 staining was also performed (lung), as shown previously [19]. Vectastain ABC-PO KIT for mouse IgG (Vector Laboratories, Burlingame, CA) was employed for the avidin–biotin complex (ABC) method. Mouse monoclonal antibodies were diluted to 1:500 for PCNA (Dako Japan, Kyoto, Japan) or 1:1000 for cyclin D1 (Santa Cruz Biotechnology, Santa Cruz, CA) and incubated at 4 °C overnight. Then they were treated with biotin-labeled mouse IgG, ABC reagent, and visualized by 3,3′-diaminobenzidine tetrahydrochloride (DAB; Wako Pure Chemical Industries) with counter-staining by hematoxylin. To determine the PCNA and cyclin D1-positive indices in the lung, more than 1000 cells were counted in the randomly selected areas of normal alveolar epitheliums. To determine the PCNA positive index in the colon, at least 1000 epithelial cells were counted in the areas of well-visualized crypts from the proximal, middle and distal colonic portions. For all cases, indices were expressed as the percentages of positively-stained cells per totally-counted cells.

2.7. Immunohistochamistry for GST-P

In order to assess the rat liver pre-neoplastic lesions, sections ($n = 18–20$ in groups 1–3; $n = 5$ in groups 4 and 5) from three liver lobes (left, intermediate and caudate) were immunohistochemically stained for GST-P using anti-rabbit GST-P polyclonal antibodies (Medical & Biological Laboratories, Nagoya, Japan) at 1:1000 dilution, as described previously [17]. The numbers and the areas of GST-P foci 0.2 mm or more in diameter and the total areas of the HE-stained liver sections were measured with the aid of the Image Processor for Analytical Pathology (IPAP) system (Sumica Technos, Osaka, Japan).

2.8. Statistical analysis

Numerical values expressed as means ± SD were subjected to the *F*-test followed by the Student's or Welch's *t*-tests using StatLight software (Yukms Co, Tokyo, Japan). Differences in incidences of neoplastic and pre-neoplastic lesions were analyzed by the χ^2 or Fisher's exact provability test using Stat-View software (SAS Institute, Cary, NC). A *P* value of less than 0.05 was considered to be statistically significant.

3. Results

3.1. Animal condition, final body and organ weights, and intakes of water and diet

Although the modified DMBDD treatment conducted in this study was toxic, only 3 out of 60 animals became moribund or were found dead due to tumors. The remaining 57 DMBDD-treated rats were healthy until the end of the regimen at week 30, like the non-DMBDD-treated animals. Therefore, PJJ-34 did not affect survival of animals within this period.