Short Communication

The *CYP19* Gene Codon 39 *Trp/Arg* Polymorphism Increases Breast Cancer Risk in Subsets of Premenopausal Japanese

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Abstract

The production of estrogen from androgen via the estrogen biosynthesis pathway is catalyzed by aromatase *P*450 (*CYP19*). To assess the association between breast cancer risk and a polymorphism at codon 39 *Trp/Arg* of the encoding gene, a case-control study was conducted at Aichi Cancer Center Hospital in Japan. Subjects were 248 histologically confirmed breast cancer patients and 603 hospital controls without cancer. Odds ratios (OR) and 95% confidence intervals (95% CI) were determined by logistic regression analysis. The allele frequency among controls was 3.8% for the *C* allele, and the OR (95% CI) of the polymorphism relative to *TT* genotype was 1.21 (0.69-2.14) for *TC/CC* genotypes combined. There was no association between *CYP19* gene polymorphism and breast cancer risk in the study group as a whole, but homozygous and heterozygous carriers of the variant *Arg* allele showed a significantly increased risk of breast cancer among premenopausal women with a late age at first full-term pregnancy (OR 7.31, 95% CI 1.88-28.5) or a high body mass index (OR 2.77, 95% CI 1.12-6.87). Additional larger studies should be done to confirm that the rare *CYP19* variant increases the risk of breast cancer among premenopausal Japanese women. (Cancer Epidemiol Biomarkers Prev 2004;13(8):1407–11)

Introduction

Many gene polymorphisms of enzymes, ligands, and receptors potentially related to carcinogenesis have been examined for breast cancer risk, but only few have showed positive associations. It is widely accepted that estrogens are involved in the development of breast cancer and that increased lifetime exposure to endogenous estrogen is known to increase risk. Epidemiologic studies have identified several factors affecting this parameter, including early age at menarche, nulliparity, late age at menopause, late age at first full-term pregnancy, and postmenopausal obesity. Excess body fat is thought to increase postmenopausal breast cancer risk by increasing bioavailable estrogen as a result of increase in extra-ovarian estrogen production (1, 2) as well as causing change in estrogen-protein binding (3). Consequently, the enzymes involved in the biosynthesis and metabolism of estrogens (*CYP17*, *CYP19*, *CYP2D6*, *COMT*, or *CYP1A1*) have been main targets in attempts to identify genetic polymorphisms contributing to breast cancer risk.

The *CYP19* gene, located on chromosome 15, encodes the enzyme *P*450 aromatase, which catalyzes three consecutive hydroxylation reactions converting C19 androgens to aromatic C18 estrogenic steroids. Genetic variation at this locus may alter aromatase activity and thereby affect hormone levels. Two polymorphic sites have primarily been the focus of breast cancer studies: (1) a tetranucleotide repeat polymorphism ($[TTTA]_n$) in intron 4 of the *CYP19* gene (4) and (2) a *C826T* variation in exon 7, which gives rise to an $Arg^{264}Cys$ amino acid substitution that can be detected by sequencing and single-strand conformational polymorphism analysis (5-8). Kristensen et al. (9) found that the $[TTTA]_{12}$ allele was overrepresented in breast cancer cases, but this was refuted by Siegelmann-Danieli and Buetow (10). Furthermore, in a British case-control study, Baxter et al. (11) observed that $[TTTA]_{12}$ frequency of the allele was not significantly elevated in the study group as compared with the controls and concluded that the *CYP19* intron 4 $[TTTA]_n$ repeat is unlikely to have a functional effect on aromatase activity.

Miyoshi et al. (12) have identified two novel polymorphisms in the *CYP19* gene and showed that one of them (codon 39 *Trp/Arg*) is significantly associated with breast cancer risk among Japanese. The incidence rates of female breast cancer in most Asian countries are much lower than those in Western countries, and the age

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distribution in Japanese women is entirely different from that in Western countries (13), i.e., the age trend falls after menopause, whereas the age-dependent elevation of risk in premenopausal women is similar. To confirm the effect of the codon polymorphism on breast cancer risk, we undertook a case-control study with the use of data from the Hospital-Based Epidemiologic Research Program at Aichi Cancer Center (HERPACC), Japan, with especially attention to the menopausal status.

Materials and Methods

Study Subjects. As we have reported in detail (14-17), the HERPACC, featuring a self-administered questionnaire completed by first-visit outpatients at the Aichi Cancer Center Hospital (ACCH), has been ongoing for some time. Before diagnosis, subjects completed a questionnaire with items concerning family history of cancer, age at menarche and menopause, reproductive history, height, and weight. The subjects in the present study were enrolled between November 2000 and September 2002 in the framework of HERPACC. Among women referred to the Department of Breast Surgery in ACCH, cases were female breast cancer patients ages 30 to 78 years histologically confirmed at ACCH. Controls were 603 female first-visit outpatients who visited the Department of Breast Surgery during same period and had never been diagnosed as having cancer. After providing informed consent, study subjects donated a 7 mL sample of peripheral venous blood on the first-visit day.

Laboratory Methods. DNA was extracted from buffy coat fractions using a QIAamp DNA Blood Mini Kit (Qiagen, Inc., Valencia, CA). Genotyping was conducted by a new method, PCR with confronting two-pair primers (18), amplification for *CYP19* being achieved using primers F1: 5′-ATCTGTACTGTACAGCACC-3′ and R1: 5′-ATGTGCCCTCATAATTCCG-3′ for the *C* (*Arg*) allele and F2: 5′-GGCCTTTTTCTCTTGGTGT-3′ and R2: 5′-CTCCAAGTCCTCATTTGCT-3′ for the *T* (*Trp*) allele. Genomic DNA (30 to 100 ng) was added to 25 μL of reaction medium with 0.15 mmol/L deoxynucleotide triphosphates, 25 pmol of each primer, 5 units AmpliTaq Gold, and 2.5 μL GeneAmp 10× PCR buffer including 15 mmol/L $MgCl_2$ (Perkin-Elmer, Foster City, CA). Amplification conditions were 10-minute initial denaturation at 95°C followed by 30 cycles of 1 minute at 95°C, 1 minute at 54°C, and 1 minute at 72°C and 5-minute final extension at 72°C. The amplified DNA was visualized on 2% agarose gel with ethidium bromide staining. Genotypes were distinguished as follows: a 200-bp band for the *T* allele, a 264-bp band for *C* allele, and a 427-bp common band as shown in Fig. 1.

Both estrogen receptor (ER) and progesterone receptor (PR) levels in breast tissue were determined using an enzyme immunoassay commercial kits. In this study, the receptor status was known for 83.1% of cases, with ER and PR positive rates of 66.0% and 59.5%, respectively.

Statistical Analysis. Logistic regression was used to obtain odds ratios (OR) and 95% confidence intervals (95% CI) as estimates of relative risk. Calculations were done using the SAS logistic procedure (SAS Institute, Cary, NC). Body mass index (BMI) was calculated as weight (kg)/height $(m)^2$. The probability for Hardy-Weinberg equilibrium was examined by Stata (College Station, TX).

Results

The characteristics of the subjects are shown in Table 1. The age distribution was slightly lower in controls than in cases, whereas the latter had a statistically significant later age at first full-term pregnancy. Compared with controls, women with breast cancer were more likely to report a family history of breast cancer. Because nutritional conditioning was dramatically changed after the World War II in Japan, the age at menarche varies with generation. Among control group, the mean ages of menarche were 12.7 years in premenopausal women and 13.7 years in postmenopausal women. When we stratified by menopausal status, the age-adjusted OR (95% CI) was 0.95 (0.56-1.61) for age at menarche ≥14 years relative to age at menarche ≤12 years in premenopausal women. Later age at menarche lowered the risk of breast cancer in postmenopausal women (OR 0.88, 95% CI 0.42-1.81 for age at menarche ≥16 versus ≤14 years).

As shown in Table 2, the allele frequencies for the mutated type were 4.4% and 3.8% for cases and controls, respectively. For genotyping quality control, we conducted retyping with other method (PCR-RFLP method) and confirmed the complete agreement of genotyping. The age-adjusted OR (95% CI) for *TC/CC* genotypes combined was 1.21 (0.69-2.14) with reference to *TT* genotype (Table 3). There were no substantial differences in the estimated ORs for the premenopausal and postmenopausal groups. The corresponding ORs (95% CI) for women with and without family history were 0.77 (0.14-4.22) and 1.23 (0.68-2.23), respectively. The ORs were not significant for the subgroups stratified by age at

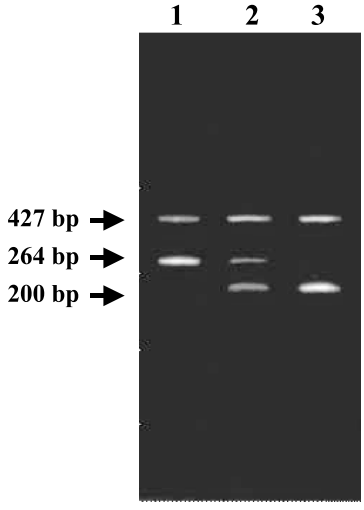

Figure 1. Gel showing the three genotypes for the *CYP19* gene codon 39 *Trp/Arg* polymorphism. *Lane 1, CC* genotype (427 and 264 bp); *lane 2, TC* genotype (427, 264, and 200 bp); and *lane 3, TT* genotype (427 and 200 bp).

Table 1. Characteristics of the subjects

Characteristics	Cases (n = 248), n (%)	Controls (n = 603), n (%)
Age (y) at diagnosis for cases or at interview for controls		
20-29	3 (1.2)	59 (9.8)
30-39	28 (11.3)	135 (22.4)
40-49	68 (27.4)	187 (31.0)
50-59	80 (32.3)	147 (24.4)
60-69	52 (21.0)	61 (10.1)
70-79	17 (6.9)	14 (2.3)
Menopausal status		
Premenopausal	115 (46.4)	385 (63.9)
Postmenopausal	133 (53.6)	218 (36.2)
BMI		
Premenopausal women		
<20	45 (39.1)	148 (38.2)
20-24	42 (36.5)	188 (48.6)
>24	28 (24.4)	51 (13.2)
Postmenopausal women		
<20	17 (12.8)	33 (15.1)
20-24	70 (52.6)	100 (45.9)
>24	46 (34.6)	85 (39.0)
Age at menarche (y)		
≤12	66 (26.9)	235 (39.7)
13-14	119 (48.6)	261 (44.1)
≥15	60 (24.5)	96 (16.2)
Age at first full-term pregnancy (y)		
≤23	78 (31.5)	263 (43.9)
24-26	84 (33.9)	165 (27.6)
≥27	86 (34.7)	171 (28.6)
Age-adjusted percentage for family history of breast cancer*	10.5	8.1
HRT		
Never use	235 (95.5)	547 (93.8)
Ever use	4 (1.6)	23 (4.0)
Unknown	7 (2.9)	13 (2.2)

*A mother or sister(s) with breast cancer.

menarche, parity, and age at first full-term pregnancy among postmenopausal women. In the premenopausal women, an elevated OR of carriers (homozygous and heterozygous) of the *C* allele was found for those with late age at first full-term pregnancy (≥26 years; OR 7.31, 95% CI 1.88-28.5).

When stratified by BMI level, the rare *CYP19* variant increased the risk of breast cancer among premenopausal women with a high BMI. The age-adjusted OR (95% CI) was 2.77 (1.12-6.87) for premenopausal women with a BMI ≥ 21. A nonsignificant trending for decrease in the OR in carriers of the *C* allele was found for postmenopausal women with BMI ≥ 24 (OR 0.74, 95% CI 0.18-3.03). We analyzed the association between *CYP19* genotype and breast cancer risk by hormone receptor status, but the OR was not significant for subgroups stratified for ER and PR. Age-adjusted ORs (95% CI) for *TC/CC* genotypes combined were 1.67 (0.74-3.76) among ER negative group and 0.88 (0.41-1.89) among ER positive group. The corresponding ORs (95% CI) for negative and positive PR were 1.34 (0.59-3.03) and 1.01 (0.47-2.17), respectively.

Table 2. Details for *CYP19* allele frequency and genotype

Codon 39 *Trp/Arg*	Allele frequency		Genotype		
	T, n (%)	*C*, n (%)	*TT*, n (%)	*TC*, n (%)	*CC*, n (%)
Cases	474 (95.6)	22 (4.4)	227 (91.5)	20 (8.1)	1 (0.4)
Controls	1,160 (96.2)	46 (3.8)	561 (93.0)	38 (6.3)	4 (0.7)

Table 3. Association between *CYP19* genotype and breast cancer risk among Japanese women overall

	Cases, n (%)	Controls, n (%)	Age adjusted OR (95% CI)
All women			
TT	227 (91.5)	561 (93.0)	1.00 (reference)
TC, CC	21 (8.5)	42 (7.0)	1.21 (0.69-2.14)
Premenopausal women			
TT	103 (89.6)	360 (93.5)	1.00 (reference)
TC, CC	12 (10.4)	25 (6.5)	1.63 (0.77-3.44)
Postmenopausal women			
TT	124 (93.2)	201 (92.2)	1.00 (reference)
TC, CC	9 (6.8)	17 (7.8)	0.83 (0.35-1.95)

Discussion

Before drawing conclusions from the present study, certain potential limitations should be considered. Quality control is an important issue for genotyping. To assess the statistical quality control, we used the Hardy-Weinberg test. The allelic distribution for controls was derived from PCR with confronting two-pair primers method departure from Hardy-Weinberg frequency (exact P value = 0.008). Accordingly, we conducted regenotyping with other method (PCR-RFLP method) and confirmed the complete agreement of genotyping. Furthermore, the identification of genotype was done with double-blind checked, and the results of genotyping were loaded into the computer by another two data researchers. Although the allelic distribution applied to PCR with confronting two-pair primers method did not fit the Hardy-Weinberg frequencies, it may be due to the fluctuation of *CC* genotyping. As a matter of fact, the estimated allele frequency (*TT*, *TC*, and *CC*) by Hardy-Weinberg frequencies was 557.88, 44.25, and 0.88, respectively; meanwhile, observed distribution of genotyping by PCR with confronting two-pair primers was 561 (93.0%), 38 (6.3%), and 4 (0.7%), respectively.

Other potential limitations of the present study should be considered. One methodologic issue is possible bias due to use of hospital-based noncancer patients as referents. In Japan, outpatients, in general, visit hospitals directly when they have symptoms and/or some anxiety about their health condition. This situation is very different from that in the United States, where people visit local general clinics first and are then referred to hospitals that function as secondary and/or specific facilities for further medical treatment. At ACCH, incident cancer cases comprised only 13% of all new outpatients in women. Among all the first-visit outpatients between January 1988 and December 2000 (n = 82,552), 64,501 (78.1%) subjects were noncancer patients. In ACCH, 70% of noncancer patients were disease free. Apparently,

there are some different characteristics from those of the general population. To evaluate this issues in using first-visit outpatients as referents in epidemiologic studies, we conducted a study that included 1,231 subjects randomly selected from the Nagoya electoral roll. We confirmed that it is feasible to use noncancer outpatients as referents in epidemiologic studies (19).

The present case-control study of the codon 39 *Trp/Arg* polymorphism of *CYP19* gene showed no association with breast cancer risk among the whole study group, but links were noted for premenopausal homozygous and heterozygous carriers of the variant allele *Arg* that significantly increased risk of breast cancer among women with a later age at first full-term pregnancy (OR 7.31, 95% CI 1.88-28.5). Premenopausal women carriers of the variant allele with a BMI $\geq$ 21 showed a significant increased risk (OR 2.77, 95% CI 1.12-6.87).

It is unclear at present why codon 39 variant carriers showed a higher frequency in premenopausal cases with later age at first full-term pregnancy or premenopausal cases with greater BMI. It is well documented that obesity is a risk factor for postmenopausal breast cancer but not for premenopausal breast cancer, and we have also addressed these issues using the HERPACC data (16). If the results from the present study are not by chance, this polymorphism may be associated with breast cancer risk at reproductive age via aromatase activity at key branch points in steroidogenesis.

The presence of both ER and PR in breast cancer tissues has been recognized as an important prognostic factor for the clinical course. However, it is not yet clear whether mammary neoplasms with differing hormone receptor status represent etiologically distinct forms of the disease with different risk factor profiles. In our previous study with HERPACC data, some evidence was obtained that certain risk factors, including reproductive variables, may differ with the PR but not the ER status (20). In the present study, the OR was not significant for the subgroups stratified by ER and PR status. Due to the insufficient number of cases, further stratification for both receptors combined and the menopausal status could not be conducted. The possibility that the frequency of codon 39 variant carriers differ with the hormone receptor status should be further pursued in future large-scale studies.

Although a polymorphism in *CYP19* in intron 4 $[TTTA]_n$ repeat alleles been reported to be associated with breast cancer risk among Caucasian women, conflicting evidence has been published. A possible role has been suggested by Haiman et al. (21) and Kristensen et al. (9) who reported an increased risk for carriers of $[TTTA]_{12}$ alleles. However, another group reported a statistically significant inverse association for this allele (10, 11). Haiman et al. (21) also found a significantly increased risk for carriers of the rare $[TTTA]_{10}$ allele in the nested case-control study within the Nurses' Health Study cohort; but again, this finding has not been confirmed by others (10, 22). Baxter et al. (11) concluded that the *CYP19* intron 4 $[TTTA]_n$ repeat is unlikely to have a functional effect on aromatase activity, and it is more likely that the $[TTTA]_8$ and $[TTTA]_{10}$ variants are in linkage disequilibrium with other functional *CYP19* variants (11). In the case-control study conducted in Japan, homozygous carriers of the allele with $\geq$10 TTTA repeats at intron 4 showed a tendency for elevated (OR 1.80, 95% CI 0.97-3.36) breast cancer risk (12). Thus, the association remains somewhat equivocal.

Regarding other genetic variation at the candidate locus, a base change from cytosine by thymine in exon 7 of the *CYP19* gene has been observed, resulting in a single amino acid substitution of Cys for Arg at codon 264 (5, 6). Watanabe et al. (8) reported no statistically significant association between the *Arg/Cys* polymorphism at codon 264 and the increased risk of Japanese breast cancer, and it does not affect aromatase activity. Although the effect of *CYP19* gene codon 39 *Trp/Arg* polymorphism on breast cancer risk was limited for subsets of premenopausal Japanese women, this finding from the present study suggested that *CYP19* gene codon 39 *Trp/Arg* polymorphism may modify the risk of Japanese female breast cancer through estrogen biosynthesis. Metabolism of estrogens is also regulated by other genes such as *CYP11A*, *CYP17*, *EDH17B2*, *CYP1A1*, *CYP1A2*, *CYP1B1*, *CYP3A4*, and *COMT*; therefore, the inconsistency with the previous finding (12) underscores the complexity of gene polymorphisms and biosynthesis of estrogen. Although our finding was not completely consistent with the previous study and we cannot conclude whether this discrepancy is because of the study population or the study itself, the *Trp/Arg* polymorphism at codon 39 of *CYP19* may modify the breast cancer risk among Japanese premenopausal women. Additional larger studies of this association are now needed for confirmation.

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