Genetic Polymorphism of Cytochrome P450–1B1 and Risk of Breast Cancer[1]

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Abstract

Cytochrome P450–1B1 (CYP1B1) is a major enzyme catalyzing the formation of genotoxic 4-hydroxyestradiol. This enzyme is also involved in the activation of polycyclic aromatic hydrocarbons and heterocyclic aromatic amines, mammary carcinogens in experimental animals. CYP1B1 is genetically polymorphic, and the variations in the *CYP1B1* gene may be related to the risk of breast cancer. We evaluated this hypothesis among 186 breast cancer cases and 200 age-matched controls as part of a large population-based case-control study conducted in urban Shanghai during 1996 to 1998. Genomic DNA from cases and controls was analyzed for genetic polymorphism in codon 432 (*Val→Leu*) of the *CYP1B1* gene using a PCR-RFLP-based assay. The frequency of the *Leu* allele was 53% in cases and 46% in controls ($P = 0.06$). Compared with those with the *Val/Val* genotype, women with the *Leu/Leu* genotype had a 2.3-fold [95% confidence interval (CI), 1.2–4.5] elevated risk of breast cancer after adjusting for potential confounding variables. This positive association was more pronounced among postmenopausal women (Odds ratio, 3.1; 95% CI, 1.0–9.1) than premenopausal women (OR, 1.9; 95% CI, 0.8–4.3). Elevated risks of breast cancer associated with homozygosity for the *Leu* allele were observed in virtually all subgroups of women defined by major risk factors for breast cancer. The results from this study were consistent with recent findings from *in vitro* and animal experiments implicating a potentially important role of CYP1B1 in the etiology of human breast cancer.

Introduction

CYP1B1[3] has been identified recently as one of the most important enzymes catalyzing the formation of 4-hydroxyestradiol (1, 2), a catechol estrogen metabolite that retains significant estrogenic activity (1, 2). This estrogen metabolite can also undergo metabolic redox cycling to generate potentially mutagenic free radicals that may damage DNA and other cell structures (2, 3). In the hamster kidney tumor model, 4-hydroxyestradiol has been shown to be carcinogenic, whereas 2-hydroxyestrogens, another major group of catechol estrogen metabolites, do not induce any tumor (2, 3). Although estrogen 4-hydroxylation is a relatively minor pathway in the liver for the formation of catechol estrogens, significant estrogen 4-hydroxylase activity has been observed in several extrahepatic estrogen target tissues (2–5), including human mammary epithelial cells (2–5). The activity of CYP1B1 was found to be higher in breast cancer than its adjacent normal tissue (6), suggesting that this enzyme may be involved in the pathogenesis of breast cancer.

Catechol estrogens, including 4-hydroxyestradiol, are metabolically inactivated by catechol-*O*-methyltransferase (COMT; Ref. 3). Recently, a variant *COMT* allele that is associated with reduced enzyme activity has been linked to elevated risk of breast cancer in several epidemiological studies (7–9), including our study among Chinese women in Shanghai (9). This suggests further that 4-hydroxyestradiol, and thus its metabolic enzyme CYP1B1, may be involved in the etiology of breast cancer.

In addition to catalyzing the formation of 4-hydroxyestradiol, CYP1B1 has also been shown to be involved in the metabolic activation of certain environmental procarcinogens, including polycyclic aromatic hydrocarbons and heterocyclic aromatic amines (10, 11), potent mammary carcinogens in experimental animals (12–14). In some experiments, CYP1B1 has been shown to be even more active than CYP1A1 in the activation of several polycyclic aromatic hydrocarbons to genotoxic intermediates (11). The expression of the *CYP1B1* gene in human breast epithelial cells suggests that this enzyme may play an important role in the *in situ* activation of both environmental carcinogens and endogenous estrogens.

The *CYP1B1* gene is located in chromosome 2p21-p22 and contains three exons (15–17). The entire coding sequence of the genes, however, is contained in exons 2 and 3 (15–17), and exon 3 encodes the heme-binding region of the enzyme (18). A G-to-C transversion at exon 3 was reported to result in a valine (**G**TG) to leucine (**C**TG) substitution in codon 432 (17). This change creates an *Eco57*I restriction site, making detection of this polymorphism in large epidemiological studies cost efficient. Prompted by the discovery of this easily identifiable polymorphism and findings from recent laboratory studies showing an important role of CYP1B1 in the metabolic activation of estrogens and environmental mammary carcinogens, we evaluated the relation between *CYP1B1* genotype and breast cancer risk in a subset of women who participated in the Shanghai Breast Cancer Study.

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[3] The abbreviations used are: CYP1B1, cytochrome P450–1B1; OR, odds ratio; CI, confidence interval; ER, estrogen receptor; PR, progesterone receptor.

Materials and Methods

The Shanghai Breast Cancer Study is a population-based case-control study conducted among Chinese women in Shanghai, the largest city on the east coast of China with a population of over six million residents (19). This study was designed to recruit all eligible breast cancer cases who were 25 to 64 years of age and newly diagnosed with breast cancer during the period of August 1996 to March 1998, as well as a representative random sample of controls from the general population. All cases and controls were permanent residents of urban Shanghai who had no prior history of cancer and were alive at the time of interview. Through a rapid case-ascertainment system, supplemented by the population-based Shanghai Tumor Registry, 1602 eligible breast cancer cases were identified during the study period, and in-person interviews were completed for 1459 (91%) of them. The major reasons for nonparticipation were refusal (109 cases, 6.8%), death prior to interview (17 cases, 1.1%), and inability to locate (17 cases, 1.1%). Cancer diagnoses for all patients were confirmed by two senior study pathologists through the review of tumor slides.

Controls were randomly selected from the female general population and frequency-matched to cases by age (5-year intervals). The number of controls in each age-specific stratum was determined in advance according to the age distribution of the incident breast cases reported to the Shanghai Tumor Registry during 1990–1993. The Shanghai Resident Registry, which keeps registry cards for all adult residents in urban Shanghai, was used to randomly select controls. For each age-predetermined control, a registry card identifying a potential control of the same 5-year age group was randomly selected. Only the women who lived at the address during the study period were considered to be eligible for the study. In-person interviews were completed for 1556 (90.3%) of the 1724 eligible controls identified. Reasons for nonparticipation included refusal (166 controls, 9.6%) and death or a prior cancer diagnosis (2 controls, 0.1%).

A structured questionnaire was used to elicit detailed information on demographic factors, menstrual and reproductive history, hormone use, dietary habits, prior disease history, physical activity, tobacco and alcohol use, weight, and family history of cancer. All participants were also measured for their current weight, circumferences of the waist and hip, and sitting and standing heights. Among those who completed the in-person interviews, blood samples, 10 ml from each woman, were collected using EDTA or heparin Vacutainer tubes from 1193 (82%) cases and 1310 (84%) controls. These samples were processed on the same day at the Shanghai Cancer Institute, and buffy coats (WBCs) for each participant were aliquoted into two 2-ml vials and stored −70°C.

Only a subset of subjects (200 cases and 200 controls) was included in the current study to better use the limited funds available for this substudy. With this sample size, we had 85% statistical power to detect a 2-fold or higher risk of breast cancer for factors with a 20% frequency (type 1 error, 0.05, two-sided). The subjects included in this study were primarily from those who were recruited in the early phase of the Shanghai Breast Cancer Study, because the genotyping work was initiated prior to the completion of the main study. Fourteen patients with a diagnosis of benign breast disease were accidentally included in the initial case group and were subsequently excluded from the analysis. Genomic DNA for cases and controls was extracted from buffy coat fractions using the method described previously (20). Briefly, frozen WBCs were thawed at the room temperature and then digested overnight at 55°C in 500 μl lysis buffer [50 mM Tris-HCl (pH 8.5), 1 mM EDTA, 0.2% SDS, and 200 μg/ml proteinase K]. The digestion was precipitated directly with isopropanol, and the pellets were washed with 70% ethanol. The genomic DNA pellets (50–100 μg) were dissolved in 300–800 μl of Tris-EDTA buffer. Genomic DNA (10–100 ng) was used for each PCR reaction.

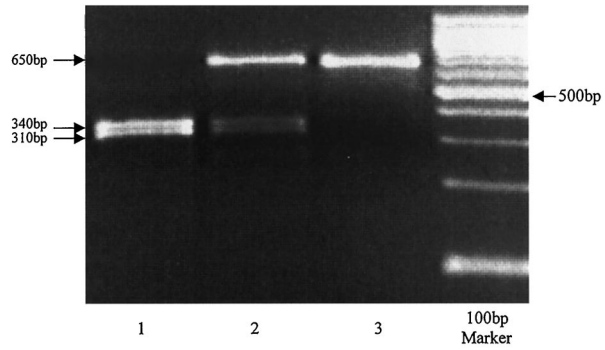

Fig. 1. Gel electrophoresis after RFLP analysis of the 650-bp *CYP1B1* PCR product. Representative genotypes are shown in *Lane 1* (homozygous for the *Leu* allele), *Lane 2* (heterozygous for the *Leu* allele), and *Lane 3* (homozygous for the *Val* allele).

CYP1B1 genotypes were determined using a PCR-RFLP-based assay. According to the published sequence of the human *CYP1B1* gene, we designed two primers (forward, 5′-TCACTTGCTTTTCTCTCTCC; reverse, 5′-AATTTCAG-CTTGCCTCTTG) to amplify a 650-bp fragment of exon 3. The PCR reactions were performed on Perkin-Elmer GeneAmp System 9700 according to the manufacturer's protocol. Specifically, these reactions were carried out in 50 μl of volume of 20 mM Tris-HCl (pH 8.4), 50 mM KCl, 1.0 mM $MgCl_2$, 0.2 mM deoxynucleotide triphosphate, 1 unit of *Taq* polymerase, and 0.4 μM of each oligonucleotide primer. The reactions were heated to 94°C for 1 min, followed by 35 cycles of 94°C for 30 s, 60°C for 30 s, and 72°C for 40 s. At the end, the reactions were extended for 7 min at 72°C. Each PCR product was subjected to *Eco57*I digestion prior to electrophoresis. The DNA fragments were then separated using 3% 2:1 Nusiev/SeaKern agarose gel. The allele types were determined as follows: two fragments of 310- and 340-bp for the *Leu* allele (**C**TG), and a single 650-bp fragment for the *Val* allele (**G**TG). Representative genotypes are shown in Fig. 1. To monitor the quality of laboratory work, a second tube of 20 study samples was included in the genetic assays. Laboratory staff were blinded to the identification of the quality control samples. The genotypes for 20 women that were determined from the first and second tubes were in complete agreement.

ORs were used to measure the strength of the association between *CYP1B1* genotypes and cancer risk (18). Stratified analyses were conducted to examine the potential confounding or modifying effect of other risk factors in the *CYP1B1*-breast cancer association. Unconditional logistic regression was used to control for potential confounders and derive adjusted ORs and 95% CIs. All statistical tests were based on two-tailed probability.

Results

Table 1 compares the distributions of cases and controls by selected demographic characteristics and major risk factors for breast cancer. Breast cancer cases and controls were comparable in age and education level. With the exception of family history of breast cancer, positive associations were observed for all major risk factors for breast cancer, although the ORs were statistically significant only for a prior history of breast fibro-

Table 1 Comparison of cases and controls by selected demographics and breast cancer risk factors, Shanghai Breast Cancer Study, 1996–1998

	Cases (n = 186)[a]	Controls (n = 200)[a]	OR (95% CI)
Demographic factors			
Age (yr)	47 (42, 53)[b]	46 (41, 55)[b]	
Education ≥ high school	40.3	43.0	0.9 (0.6–1.3)
Major risk factors			
First-degree relative with breast cancer	1.6	2.0	0.8 (0.2–3.6)
Ever diagnosed with breast fibroadenoma	9.7	4.0	2.6 (1.1–6.1)
No regular leisure physical activity	80.1	69.0	1.8 (1.1–2.9)
Waist:hip ratio ≥0.85	21.6	18.5	1.2 (0.7–2.0)
Body mass index ≥25	27.0	22.5	1.3 (0.8–2.0)
Age at menarche ≤12 yr	9.2	7.5	1.2 (0.6–2.6)
Age at menopause ≥50 yr[c]	50.0	38.0	1.6 (0.8–3.2)
Age at first live birth ≥30 yr[d]	25.6	16.0	1.8 (1.1–3.0)

[a] Unless otherwise specified, percentages are presented.
[b] Median (25th, 75th percentile) are presented.
[c] Among postmenopausal women.
[d] Among parous women.

Table 2 Associations of *CYP1B1* genotype with breast cancer risk, Shanghai Breast Cancer Study, 1996–1998

CYP1B1 genotype	No. of cases (n = 186)	No. of controls (n = 200)	Unadjusted OR (95% CI)	Adjusted[a] OR (95% CI)
All study participants				
Val/Val	37	44	1.0	1.0
Val/Leu	101	127	0.9 (0.6–1.6)	1.0 (0.6–1.7)
Leu/Leu	48	29	2.0 (1.0–3.7)	2.3 (1.2–4.3)
Premenopausal women				
Val/Val	26	26	1.0	1.0
Val/Leu	68	78	0.9 (0.5–1.6)	1.0 (0.5–1.9)
Leu/Leu	27	17	1.6 (0.7–3.6)	1.9 (0.8–4.3)
Postmenopausal women				
Val/Val	11	18	1.0	1.0
Val/Leu	32	49	1.1 (0.4–2.6)	1.1 (0.4–2.6)
Leu/Leu	21	12	2.9 (1.0–8.0)	3.1 (1.0–9.1)

[a] Adjusted for prior history of breast fibroadenoma, physical activity, and age at first pregnancy.

adenoma, no leisure physical activity, and late age at first live birth. These three factors were included in these multivariate analyses as potential confounders. The lack of statistically significant associations with other breast cancer risk factors may be attributable to the small sample size of this substudy.

The *Leu* allele frequency was 53% in cases and 46% in controls (P = 0.06). Twenty-six % of cases *versus* 15% of controls were found to have the *Leu/Leu* genotype in our study population, resulting in an OR of 2.3 (95% CI, 1.2–4.3) after adjusting for potential confounding factors (Table 2). This positive association was more pronounced among postmenopausal women (OR, 3.1; 95% CI, 1.0–9.1) than premenopausal women (OR, 1.9; 95% CI, 0.8–4.3). Heterozygosity for the *Leu* allele was not found to be associated with breast cancer risk.

To evaluate the consistency of the association between the *CYP1B1* genotype and breast cancer risk, stratified analyses were performed (Table 3). Because women with the *Val/Leu* genotype had similar risk of breast cancer as those with the *Val/Val* genotype, these two groups of women were combined to enhance the stability of risk estimate. With the exception of the women with a prior history of breast fibroadenoma, women with the *Leu//Leu* genotype had a higher risk of breast cancer than the group of women with combined *Val/Val* and *Val/Leu* genotypes in all strata of life-style factors presented in Table 3, indicating that the *Leu/Leu* genotype may be a risk factor independent of these life-style factors.

Discussion

Despite strong evidence from animal studies and *in vitro* experiments (1–6), the potential role of CYP1B1 in the etiology of human breast cancer has not been investigated adequately. To our knowledge, only one study published to date has evaluated the association of *CYP1B1* genetic polymorphisms with breast cancer risk (21). No statistically significant association between the *CYP1B1* polymorphisms and breast cancer risk, however, was observed in that study, a hospital-based case-control study involving 164 Caucasian and 59 African-American women with breast cancer who were treated at the Vanderbilt University Medical Center over a 14-year period from 1982 to 1996. Controls for that study were patients treated in the same hospital for diseases other than cancer. Participation rates for cases and controls were not provided, and information related to most demographic and risk factors for breast cancer was not collected. Therefore, potential biases associated with hospital-based case-control studies might be a concern for that study.

The functional significance of the $Val^{432}Leu$ polymorphism has not been studied adequately. Because exon 3 encodes for the heme-binding region of the CYP1B1 enzyme, nucleotide changes in this exon may be significant to the function of this enzyme. Bailey *et al.* (21) reported that Caucasian patients with the *Val/Val* genotype had a significantly higher percentage of breast cancer that were positive for ERs or PRs, suggesting that this polymorphism may be functionally important for the expression of these steroid receptors in breast cancer (21). Because the prognosis for ER/PR-positive cancer is better than ER/PR-negative cancer, case-control studies including prevalent cases may be subjective to survival bias. In other words, the *Val/Val* genotype may be overrepresented in prevalent cases, which may attenuate the positive association, if any, with the *Leu/Leu* genotype. In our study, all breast cancer cases were newly diagnosed, and only 1.1% of eligible cases were missed because of death prior to interviews. Therefore, the potential for survival bias should be minimal in this study. We found that the *Leu/Leu* genotype was associated with an elevated risk of breast cancer. Furthermore, the positive association between the *Leu/Leu* genotype and breast cancer was more pronounced among postmenopausal women, among whom more breast cancers are reported to be positive for ERs and/or PRs than those diagnosed among premenopausal women. We were unable at present to evaluate directly the association of *CYP1B1* genotypes with subtypes of breast cancer defined by steroid receptors, because no such data were collected.

In addition to estrogen 4-hydroxylation, estrogen hydroxylations also occur at C2, C6, C15, and C16 positions. Among them, estrogen C2 and C16 hydroxylation are of particularly biological and quantitative importance (2). Similar to 4-hydroxyestrogen, 16α-hydroxyestrone has been shown to be genotoxic, reacting with DNA to form adducts (2). On the other hand, 2-hydroxyestrogen has no estrogenic effect, and in fact, 2-methoxyestrogen may act as an antiestrogen in estrogen-sensitive tissues (2, 3). CYP1B1 primarily catalyzes estrogen 4-hydroxylation (2). We found that the positive association of the *CYP1B1-Leu* allele with breast cancer was stronger among postmenopausal than premenopausal women. Because the estrogen level is higher among premenopausal than postmenopausal women, our findings suggest that this

Table 3 Adjusted ORs for potential joint effect of *CYP1B1* genotype with selected risk factors, Shanghai Breast Cancer Study, 1996–1998

Risk factors	*CYP1B1 Val/Val + Val/Leu*		*CYP1B1 Leu/Leu*	
	Case/control	OR (95% CI)[a]	Case/control	OR (95% CI)[a]
Ever diagnosed with breast fibroadenoma				
No	123/165	1.0	45/27	2.4 (1.4–4.1)
Yes	15/6	3.2 (1.2–8.6)	3/2	2.1 (0.3–13.2)
Physical activity				
High	24/53	1.0	13/9	3.5 (1.3–9.5)
Low	114/118	2.1 (1.2–3.6)	35/20	3.8 (1.8–7.9)
Age at first live birth (by median)[b]				
≤26 years	59/93	1.0	25/16	2.6 (1.3–5.3)
>26 years	74/72	1.5 (1.0–2.5)	21/11	2.8 (1.3–6.3)

[a] With the exception of the variables under evaluation, ORs were adjusted for the other two variables listed in the table.
[b] Among parous women.

polymorphism might be more closely related to the activation of environmental mammary carcinogens than estrogen 4-hydroxylation. Very recent data from *in vitro* experiments suggested that the isozyme encoded by the *Leu* allele might have an elevated activity in the activation of mammary carcinogens but a reduced activity in estrogen 4-hydroxylation (22).

The methodological limitations of this study were few. The participation rate of this study was high, minimizing potential selection bias that is common to many case-control studies. Chinese women living in Shanghai are relatively homogeneous in ethnic backgrounds, because >98% of them are classified into a single ethnic group (Han Chinese). We have also compared the place of birth between cases and controls and found that these two groups of women were very similar. Therefore, the potential confounding effect by ethnicity in association studies of genetic biomarkers, may not be a major concern in our study. The sample size in this study was not large, which may have resulted in a relatively unstable estimate of risk, particularly in stratified analyses. Nevertheless, the results from this study are consistent with the observations from laboratory investigation, implicating a potential important role of the CYP1B1 enzyme in the etiology of breast cancer. Our findings are thus biologically plausible and merit further investigation with a larger sample size or in other ethnic populations.

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