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Association of Cytochrome P450 *1B1* (*CYP1B1*) Polymorphism with Steroid Receptor Status in Breast Cancer[1]

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Abstract

A key enzyme involved in the production of potentially carcinogenic estrogen metabolites and the activation of environmental carcinogens is cytochrome P450 1B1 (CYP1B1), the predominant member of the CYP1 family expressed in normal breast tissue and breast cancer. Because of the preeminent role of CYP1B1 in mammary estrogen/carcinogen metabolism, we examined the *CYP1B1* gene to determine whether genetic differences could account for interindividual differences in breast cancer risk. We focused on exon 3, because it encodes the catalytically important heme binding domain of the enzyme, and discovered three polymorphisms of which two are associated with amino acid substitutions in codons 432 (*Val→Leu*) and 453 (*Asn→Ser*), designated as *m1* and *m2*, respectively. Approximately 40% of Caucasian women have the *m1 Val* allele compared with nearly 70% of African-American women ($P < 0.0001$). The allele frequency also differs significantly in *m2*, with the rare *Ser* allele being present in 17.4% of Caucasians but only in 3.4% of African Americans ($P < 0.0003$). To determine whether the polymorphic *CYP1B1* alleles hold implications as potential breast cancer risk factors, we compared the *CYP1B1* genotypes in 164 Caucasian and 59 African-American breast cancer cases with those in age-, race-, and frequency-matched controls. Odds ratio calculations failed to show a significant association between any of the genotypes and breast cancer. Because CYP1B1 is known to be involved in mammary estrogen metabolism, we investigated whether the estrogen receptor status is influenced by the *CYP1B1* genotypes. Caucasian patients with the *m1 Val/Val* genotype have a significantly higher percentage of estrogen receptor-positive ($P = 0.02$) and progesterone receptor-positive breast cancers ($P = 0.003$). There was no correlation with the *m2* genotypes. These data suggest that the *CYP1B1* polymorphisms in exon 3 are not associated with increased breast cancer risk but that the *m1* polymorphism may be functionally important for steroid receptor expression in breast cancer of Caucasian patients.

Introduction

CYP1B1[3] is a key enzyme involved in the production of potentially carcinogenic estrogen metabolites and the activation of environmental carcinogens (1, 2). CYP1B1, together with CYP1A1, has been shown to catalyze the formation of mutagenic intermediates from a number of polycyclic aromatic hydrocarbons, including several that are potent mammary gland carcinogens in rodents. CYP1B1 appears to be more active than CYP1A1 in the conversion of several polycyclic aromatic hydrocarbons to genotoxic intermediates (2). CYP1B1 also exceeds CYP1A1 in its catalytic efficiency as E_2 hydroxylase, principally owing to its low K_m for E_2 (2, 3). CYP1B1 has its primary activity at the C-4 position of E_2 with a 5-fold lower activity at C-2, whereas CYP1A1 has activity at the C-2, C-6α, and C-15α positions. The E_2 4-hydroxylase activity of CYP1B1 has received particular attention due to the fact that the catechol estrogen, 4-hydroxyestradiol, is carcinogenic in animal models (4). Moreover, human breast cancer tissue was shown to have significantly higher levels of E_2 4-hydroxylation than adjacent normal breast tissue (5). CYP1B1 is expressed both in normal breast and virtually all breast cancers (6, 7). Therefore, endogenous production of potentially carcinogenic catechol estrogens as well as metabolic activation of exogenous procarcinogens by CYP1B1 place the enzyme in a key position for mammary carcinogenesis. Inherited alterations in CYP1B1 activity hold the potential to define differences in carcinogen exposure and thereby explain differences in breast cancer risk. In this study, we examined the human *CYP1B1* gene to determine whether genetic differences are associated with increased breast cancer risk.

Materials and Methods

Subjects. The study is based on 164 Caucasian women and 59 African-American women with primary invasive breast cancer who were treated at Vanderbilt University Medical Center, Nashville, TN, between 1982 and 1996. All patients except seven had tumors of sufficient size (≥1.0 cm) to allow multipoint hormone-binding analyses of ER and PR and extraction of DNA in addition to routine histopathological studies. In seven tumors, the amount of tissue was insufficient for PR analysis. Each breast cancer patient was matched by age and race to a control patient hospitalized at Vanderbilt University Medical Center for various medical reasons other than breast cancer or other forms of malignancy. Peripheral blood leukocytes served as source for DNA for the controls.

Biochemical Analysis. In the ligand binding analyses for ER and PR status, tumors were considered positive if values exceeded 10 fmol/mg. DNA was isolated from all samples using a DNA extraction kit (Stratagene, La Jolla, CA). For PCR analysis of exon 3 of the *CYP1B1* gene, we designed appropriate primers (primer 1, 5′-GTG GTT TTT GTC AAC CAG TGG; primer 2, 5′-GCC TCT TGC TTC TTA TTG GCA) to amplify genomic DNA (0.5 μg) through 30 cycles in a 100-μl volume containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 4 mM $MgCl_2$, 200 μM each of the four deoxyribonucleotides, Taq polymerase (2 units; Promega Corp., Madison, WI), and each oligonucleotide at 25 μM. Amplification conditions consisted of a denaturing step at 95°C for 1 min, annealing at 62°C for 1 min, and polymerization at 72°C for 1 min. A sample of each PCR mixture was size fractionated by electrophoresis in a 1.5% agarose gel and visualized by ethidium bromide staining. For DNA sequencing, the PCR products from 10 individuals were gel purified and subjected to the Thermo Sequenase radiolabeled terminator cycle sequencing method using [α-^{33}P]2′,3′-dideoxynucleoside-5′-triphosphate nucleotides (Amersham, Piscataway, NJ). Sequencing reactions were carried out with primer 1 and analyzed by denaturing gel electrophoresis using glycerol-tolerant gel buffer. For genotype analysis, each PCR product was subjected to two separate restriction endonuclease reactions. Digestion with *Eco57I* revealed the *m1* polymorphism with bands of 104 and 39 bp, whereas digestion with *Cac8I* identified the *m2* polymorphism with bands of 105 and 38 bp.

Statistical Methods. Allele and genotype frequencies were calculated for each of the four groups, *i.e.*, controls and cases, Caucasians and African Americans. The statistical methods used were logistic regression, contingency table χ^2, and Fisher's exact test. Models for logistic regression analysis

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[3] The abbreviations used are: CYP1B1, cytochrome P450 1B1; CYP1A1, cytochrome P450 1A1; E_2, 17β-estradiol; ER, estrogen receptor; PR, progesterone receptor.

included age as a potential confounding variable in breast cancer. We assessed the effect of heterozygous and homozygous recessive genotypes separately in a logistic model with each possibility characterized by separate indicator variables. We estimated relative risks of breast cancer by odds ratios from 2 × 2 contingency tables. Confidence intervals for these odds ratios were derived using Woolf's method by PROC FREQ of the SAS statistical analysis package (8). *Ps* for allele frequencies were computed under the assumption of Hardy-Weinberg distribution.

Results and Discussion

The *CYP1B1* gene contains three exons, but only exons 2 and 3 encode the protein (Fig. 1*a*). Analysis of the CYP1B1 amino acid sequence shows several conserved regions of ≥40% identity to CYP1A1 (9). In our search for polymorphisms, we decided to focus on exon 3 because it encodes the heme binding domain, which is essential for the catalytic function of CYP1B1. DNA sequencing identified three polymorphic sites at nucleotides 1294G→C, 1347T→C, and 1358A→G (Fig. 1*b*). Two of the polymorphisms result in amino acid changes in codons 432 (Val→Leu) and 453 (Asn→Ser), designated as *m1* and *m2*, respectively, in analogy to polymorphisms in the *CYP1A1* gene. To date, the only abnormality of the *CYP1B1* gene has been described in primary congenital glaucoma, an autosomal recessive eye disorder. Both frameshift and missense mutations were identified in affected Turkish and Saudi Arabian families, with homozygosity being associated with the disease phenotype (10, 11).

The 1294G→C transversion creates an *Eco57I* site, whereas the 1358A→G transition creates a *Cac8I* site. We used PCR amplification and restriction endonuclease digestion to characterize *CYP1B1* genotypes (Fig. 1*c*) and to determine the distribution of *CYP1B1* alleles. As shown in Table 1 for controls, ~40% of Caucasian women have the *m1* Val allele compared with nearly 70% of African-American women ($P < 0.0001$). The allele frequency also differs significantly in *m2*, with the rare *Ser* allele being present in 17.4% of Caucasians but only in 3.4% of African Americans ($P < 0.0003$). Differences in allele frequencies between Caucasian and African-American women have also been reported for polymorphisms of the

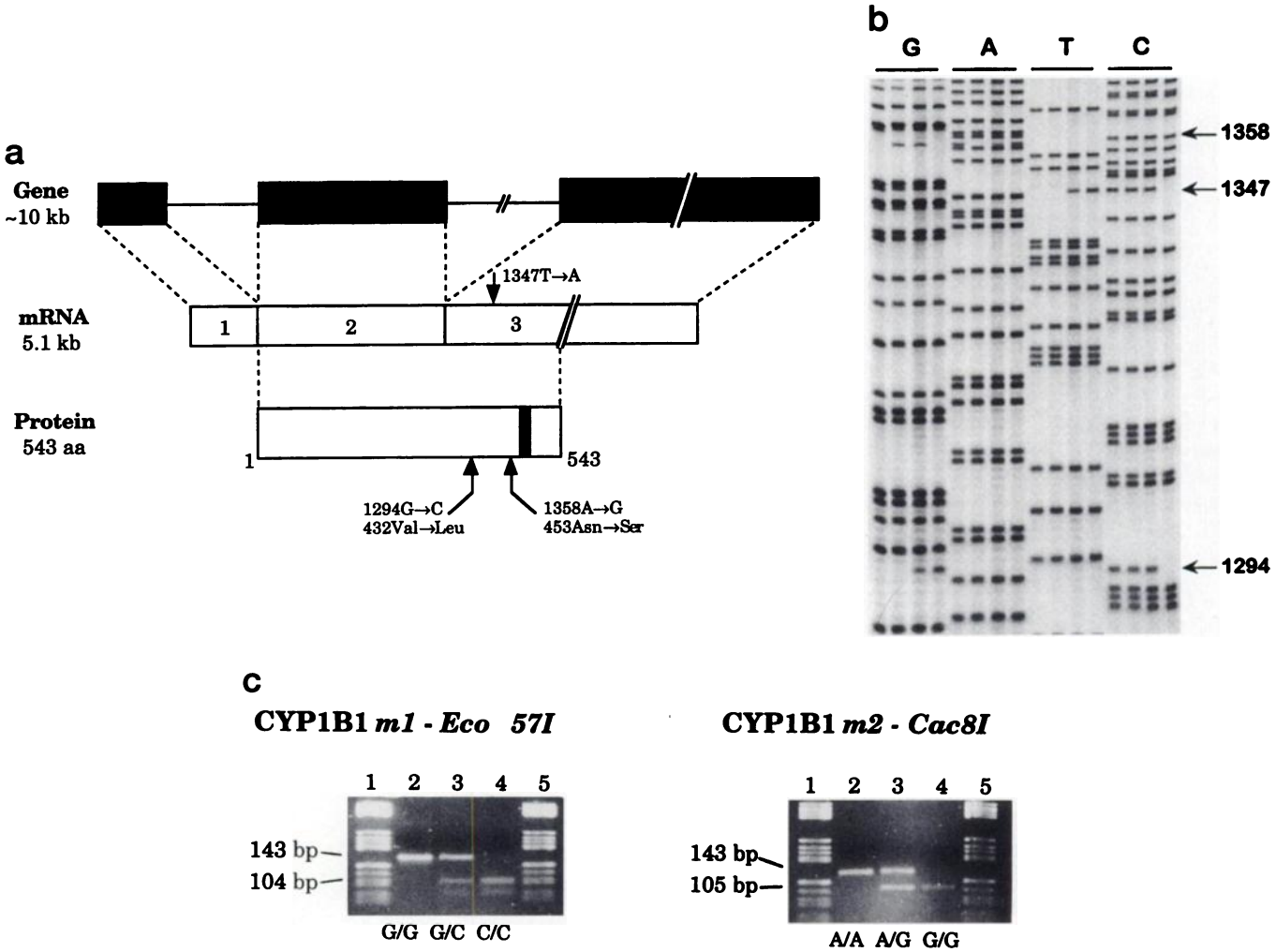

Fig. 1. *a*, overview of *CYP1B1* gene, mRNA, and protein. The *CYP1B1* gene at 2p21–22 is about 10 kb long and contains three exons. The open reading frame starts in the second exon and is 1629 bp in length, encoding a protein of 543 amino acids (*aa*). *Shaded area*, the heme-binding region. The lengths of the exons are drawn in scale, whereas those of the introns are not. Exon 3 contains three polymorphic sites at nucleotides 1294, 1347, and 1358 (*arrows*). Two of the polymorphisms result in amino acid changes in codons 432 (Val→Leu) and 453 (Asn→Ser). *b*, DNA sequence analysis of *CYP1B1* exon 3. Genomic DNA from four unrelated individuals was amplified using primers 1 (5′-GTG GTT TTT GTC AAC CAG TGG) and 2 (5′-GCC TCT TGC TTC TTA TTG GCA). Each PCR product was purified and sequenced with the Thermo Sequenase radiolabeled terminator cycle sequencing method using [α-^{33}P]2′,3′-dideoxynucleoside-5′-triphosphate nucleotides. Sequencing reactions were carried out with primer 1 and analyzed by denaturing gel electrophoresis using glycerol-tolerant gel buffer. Each set of nucleotide-specific reactions (G, A, T, and C) was grouped for the four individuals, allowing identification of polymorphic sites at 1294G→C, 1347T→C, and 1358A→G (*arrows*). *c*, analysis of *CYP1B1* genotypes by PCR amplification and restriction endonuclease digestion. Amplification of genomic DNA yielded a 143-bp PCR fragment. Digestion with *Eco57I* revealed the *m1* polymorphism with bands of 104 and 39 bp, whereas digestion with *Cac8I* identified the *m2* polymorphism with bands of 105 and 38 bp. The outside lanes contain DNA size markers. The inside *Lanes 2–4* depict the individual genotypes, as indicated beneath each lane.

Table 1 *CYP1B1* allele frequencies in Caucasian and African-American controls

Allele	Caucasian (n = 328)	African American (n = 118)	P
m1 (codon 432)			
Val	134 (40.9)[a]	82 (69.5)	<0.0001
Leu	194 (59.1)	36 (30.5)	
m2 (codon 453)			
Asn	271 (82.6)	114 (96.6)	<0.0003
Ser	57 (17.4)	4 (3.4)	

[a] Number of alleles, followed by percentage in parentheses.

CYP1A1 gene. However, the most striking differences are associated with polymorphic sites in the 3′ noncoding region of the *CYP1A1* gene (12, 13).

CYP1B1 is important for the homeostasis of estrogen in extrahepatic tissues such as the breast. To determine the relation of CYP1B1 and E_2 hydroxylation, Hayes *et al.* (1) expressed the human enzyme in *Saccharomyces cerevisiae*. Microsomes from the transformed yeast catalyzed the C-2 and C-4 hydroxylation of E_2 with K_m values of 0.78 and 0.71 mM and turnover numbers of 0.27 and 1.39 min^{-1}, respectively. Comparison with CYP1A1, CYP1A2, CYP3A3, and CYP3A4 indicates that the E_2 C-4 hydroxylase activity of CYP1B1 has the highest catalytic efficiency and the lowest K_m of all of the E_2 hydroxylases reported (3, 14). Thus, CYP1B1 appears to be the main cytochrome P450 enzyme responsible for the C-4 hydroxylation of E_2. Given the carcinogenic potential that has emerged for 4-hydroxyestradiol (15), CYP1B1 assumes a special role as the principal enzyme producing this catechol estrogen. Shimada *et al.* (2) examined the relative capacity of each member of the CYP1 enzyme family to activate carcinogenic chemicals, including polycyclic aromatic hydrocarbons, heterocyclic and aryl amines, as well as mycotoxins. Although CYP1B1 did not produce genotoxic products from benzo(*a*)pyrene or aflatoxin B_1, it had the highest catalytic activity for several procarcinogenic compounds. The involvement of CYP1B1 in the production of carcinogenic catechol estrogens as well as activation of a number of environmental carcinogens is of particular interest with respect to its high expression in benign and malignant mammary tissue (6, 7). To determine whether the variant enzymes encoded by the polymorphic *CYP1B1* alleles are associated with interindividual differences in breast cancer risk susceptibility, we compared the *CYP1B1* genotypes in 164 Caucasian and 59 African-American breast cancer cases with those in age-, race-, and frequency-matched controls. As Table 2 shows, there was no significant association between any of the genotypes and increased risk of breast cancer. These findings are similar to those seen in investigations of *CYP1A1* genotypes. Three case-control studies found no correlation between any of the four *CYP1A1* polymorphisms and breast cancer in Caucasian women with the possible exception of smokers (16, 17). A study of 21 African-American patients observed a significant correlation with the *m1* allele of CYP1A1 (18), but a larger study of 59 African-American patients found no association (13). Analysis of the menopausal status failed to show a significant association of polymorphic *CYP1B1* alleles with breast cancer in pre- or postmenopausal women for either Caucasian or African-Americans, similar to our results with the CYP1A1 polymorphisms (13).

The effects of estrogen on ER gene expression are not clear, because both positive and negative regulation have been described (19). Kaneko *et al.* (20) presented evidence that the coding region of ER cDNA possesses a sequence(s) necessary for ER down-regulation of both protein and mRNA. ER is down-regulated by E_2 in some estrogen-responsive tissues as well as in cell lines such as MCF-7 and GH3 (21). However, this effect is not true under all culture conditions of MCF-7 cells or for all breast cancer cell lines (22). Because CYP1B1 is known to be involved in mammary estrogen metabolism, we investigated whether the ER status is influenced by the *CYP1B1* genotypes. Table 3 shows that Caucasian breast cancer patients with the *m1 Val/Val* genotype have a significantly higher percentage of ER-positive tumors (P = 0.02). There was no correlation with the *m2* genotypes. The expression of PR is induced by estrogen via interaction with a functional ER. For that reason, we also examined the correlation of PR expression with the *CYP1B1* genotype. An even stronger association was found between PR positivity and the *m1 Val/Val* genotype (P = 0.003) but not with the *m2* genotypes. Evaluation of the African-American breast cancer patients failed to show a significant correlation between genotypes and receptor status (result not shown). It appears that other factors are involved in determining the ER phenotype of breast cancers in African-American women. Several groups have reported a significantly higher percentage of ER negativity in African-American than Caucasian patients (23, 24). Although CYP1A1 is also involved in mammary estrogen metabo-

Table 2 *Distribution and analysis of CYP1B1 genotypes in Caucasian and African-American breast cancer cases and controls*

	Caucasian			African American		
Genotype	Cases (n = 164)	Controls (n = 164)	Relative risk (95% CI)[a]	Cases (n = 59)	Controls (n = 59)	Relative risk (95% CI)
m1 (codon 432)						
Val/Val	27 (16.5)[b]	19 (11.6)	1.0[c]	27 (45.8)	26 (44.1)	1.0[c]
Val/Leu	85 (51.8)	96 (58.5)	0.6 (0.3–1.2)	27 (45.8)	30 (50.8)	0.9 (0.4–1.8)
Leu/Leu	52 (31.7)	49 (29.9)	0.7 (0.4–1.5)	5 (8.4)	3 (5.1)	1.6 (0.4–2.9)
m2 (codon 453)						
Asn/Asn	111 (67.7)	111 (67.7)	1.0[c]	56 (94.9)	55 (93.2)	1.0[c]
Asn/Ser	48 (29.3)	49 (29.9)	0.9 (0.6–1.6)	3 (5.1)	4 (6.8)	0.7 (0.2–3.4)
Ser/Ser	5 (3.0)	4 (2.4)	1.3 (0.3–4.8)	0	0	[d]

[a] CI, confidence interval.
[b] Number of individuals followed by percentage in parentheses.
[c] Denotes denominator for computation of odds ratios.
[d] Unable to compute odds ratio due to zero sample number in this group.

Table 3 *Analysis of CYP1B1 genotypes and ER/PR status in Caucasian breast cancer cases*

Genotype	ER-positive (n = 88)	ER-negative (n = 76)	P	PR-positive (n = 56)	PR-negative (n = 101)	P
m1 (codon 432)						
Val/Val	20 (23)[a]	7 (9)	0.02	16 (29)	10 (10)	0.003
Val/Leu or Leu/Leu	68 (77)	69 (91)		40 (71)	91 (90)	
m2 (codon 453)						
Asn/Asn	60 (68)	51 (67)	0.88	41 (73)	67 (66)	0.37
Asn/Ser or Ser/Ser	28 (32)	25 (33)		15 (27)	34 (34)	

[a] Number of breast cancers, followed by percentage in parentheses.

lism, no correlation between any of the CYP1A1 polymorphisms and ER status has been found (13). This finding is consistent with experimental data showing no functional deviation of the variant CYP1A1 enzymes (25). In contrast, the correlation between the *CYP1B1 m1* polymorphism and steroid receptor expression suggests an alteration in enzyme function for the *Val* allele. Preliminary experiments indicate that recombinant mutant *CYP1B1* has P450 catalytic activity. More detailed experiments will determine whether the polymorphisms, singly or in combination, alter CYP1B1 function with regard to estrogen and carcinogen metabolism.

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