Cytochrome P450 1B1 Gene Polymorphisms and Postmenopausal Endometrial Cancer Risk

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Abstract

Estrogen unopposed by progestins is a key factor in endometrial cancer etiology. Cytochrome P450 1B1 (CYP1B1), responsible for the 4-hydroxylation of estrogen, may be important in endometrial carcinogenesis, either as a regulator of estrogen availability or as a producer of potentially genotoxic estrogen metabolites. We investigated the association of CYP1B1 genotype and endometrial cancer risk in a population-based case-control study of postmenopausal Swedish women. We used the Expectation-Maximization algorithm to estimate the haplotype frequencies in the population and calculated odds ratios and 95% confidence intervals from conditional logistic regression models. In stratified analysis, we investigated the possible effects of CYP1B1 genotype on endometrial cancer risk in subgroups defined primarily by menopausal hormone use and also by body mass index, smoking, use of combined oral contraceptives, and family history. We genotyped 689 cases and 1,549 controls for the CYP1B1 single nucleotide polymorphisms m2, m3, and m4 and estimated the haplotype frequencies among controls to 0.086, 0.291, 0.452, and 0.169 for the CYP1B1*1, CYP1B1*2, CYP1B1*3, and CYP1B1*4 alleles, respectively. We found no evidence for an overall association between CYP1B1 genotype and endometrial cancer risk, nor was there any clear indication of gene-environment interaction. (Cancer Epidemiol Biomarkers Prev 2004;13(9):1515–20)

Introduction

Endometrial cancer is the most common gynecological cancer and occurs chiefly among postmenopausal women. The disease is classified into mainly two types. Type 1 tumors are associated with endometrial hyperplasia and accounts for ~80% of endometrial cancers, whereas type 2 tumors generally develop from atrophic endometrial tissue. Estrogens, both endogenous and exogenous in origin, play a crucial role in the etiology of endometrial cancer, particularly endometroid adenocarcinoma (type 1 tumors) (1). Risk factors include early menarche, late menopause, nulliparity, postmenopausal hormone use, and obesity (2), whereas smoking (3), use of oral contraceptives (4), and physical activity (5) seem to decrease the risk. In contrast to the breast, the effects of estrogen on endometrial cells is counteracted by progestins, which have been shown to down-regulate estrogen receptors and interfere with transcription of estrogen receptor–mediated genes (6).

Hydroxylation of estrogens is performed by cytochrome P450 enzymes, such as cytochrome P450 1B1 (CYP1B1), and in human endometrium, both 2-hydroxylation and 4-hydroxylation of estrogen have been shown (7). CYP1B1 is expressed in human endometrium (8) and the protein has been localized to the cytoplasm of epithelial glands (9). Degradation of estrogen by CYP1B1 locally in the endometrium may be of importance in endometrial cancer etiology, especially in endometrial hyperplasia. In addition to the role of CYP1B1 as an estrogen eliminator, CYP1B1 may also form potentially genotoxic catechol estrogens. The metabolite 4-hydroxyestrogen, which may be further oxidized to reactive intermediates, has been shown to induce tumors in animals (10, 11). Indeed, CYP1B1 catalyzes 4-hydroxylation of 17β-estradiol at a rate 5-fold higher than 2-hydroxylation (12). However, the impact of these metabolites in conjunction with human cancers is not known.

Several alleles of the CYP1B1 gene have been identified (http://www.imm.ki.se/CYPalleles), of which must are rare and associated with glaucoma. Four single nucleotide polymorphisms (SNP) in the CYP1B1 gene, m1, m2, m3, and m4, which cause amino acid changes, have been identified in Caucasians (13). Because m1 and m2 seem to be in complete linkage disequilibrium, there are eight possible haplotypes (see ref. 14, Table 1). Biochemical investigations of these CYP1B1 variants (CYP1B1.2, CYP1B1.3, and CYP1B1.4) by Shimada et al. (15, 16) and Watanabe et al. (17) indicate that the CYP1B1.3 variant has a higher activity toward 17β-estradiol. However, other studies report no altered enzymatic activity (18) or decreased activity (19) of CYP1B1.3.

In recent years, several studies have investigated the association of polymorphisms in estrogen biosynthesis...
and estrogen metabolism genes and the risk of hormonal cancers, such as breast and endometrial cancers. Endometrial cancer is a particularly appropriate model system for studying the effect of genetic variation of estrogen metabolism, because the development of this disease clearly is influenced by estrogen exposure. CYP1B1 polymorphisms have been studied in relation to breast cancer, by us (14) and by others (17, 20-22). In relation to endometrial cancer, one study performed on a Japanese population has been published (23). We present here results from a population-based case-control study of postmenopausal Swedish women in whom we studied CYP1B1 polymorphisms in association to endometrial cancer risk. We estimated the CYP1B1 haplotype frequencies in the population and investigated possible effects of CYP1B1 genotype on endometrial cancer risk. Subgroup analyses were done with the a priori hypothesis that any influence of CYP1B1 genotype would be more pronounced among long-term menopausal estrogen users.

Subjects and Methods

Parent Study. This nationwide, population-based case-control study encompassed all cases with incident, primary histopathologically confirmed invasive endometrial cancer among women ages 50 and 74 years residing in Sweden between January 1, 1994 and December 31, 1995 as described in detail previously (24). Endometrial cancer patients were identified at diagnosis through a notification system organized within the six Swedish regional cancer registries, to which reporting of all malignant tumors is mandatory. Controls were randomly selected in 5-year age strata (to match the expected age frequency distribution among the cases) from the Swedish Total Population Register.

Cases were asked to participate in the study by their respective physicians. We were notified of the identity and address only of consenting patients, to whom we mailed questionnaires asking for detailed information about intake of menopausal hormones and oral contraceptives, weight, height, reproductive history, medical history, and other lifestyle factors. Controls were contacted directly with the questionnaire. Only women with an intact uterus were eligible as controls. Seventy-six percent of eligible cases (n = 802) and 84% of the controls (n = 3,550) ultimately participated in the study. Among these controls, 491 who failed to return the mailed questionnaire were interviewed by phone. Results from the parent study have been published (4, 5, 24-27). Slides with tumor tissue were collected from patients, respectively, and blood samples were obtained for 603 and 104 endometrial cancer cases. Samples were coded and transferred to the laboratories. Blood samples or archived tissue samples were obtained from 1,574 control women (922 selected plus 652 from the breast cancer study), yielding participation rates of 88% for cases (707 of 802 eligible) and 76% for controls (1,574 of 2,074 eligible).

DNA was isolated from 3 ml whole blood using Wizard Genomic DNA Purification Kit (Promega, Madison, WI) according to the manufacturer’s instructions. From nonmalignant cells in paraffin-embedded tissue, we extracted DNA using a standard phenol/chloroform/isoamyl alcohol protocol (28).

Genetic Analyses. Two methods were used for CYP1B1 genotyping: multiplex fluorescent solid-phase minisequencing (hereafter called minisequencing; ref. 29), and dynamic allele specific hybridization (DASH; ref. 30). The multiplex PCR used for minisequencing was sensitive to DNA quality. To include suboptimal samples and increase genotyping success, we also used the robust high-throughput DASH method. Results from the two methods were validated with a two-step allele-specific PCR method for m2, m3, and m4 (18), 24% of the samples were analyzed with both minisequencing and DASH, and the genotypes obtained were identical. The primer and probe sequences used in the minisequencing and DASH protocols can be found in ref. 14, Table 2. All PCR reactions were done on a Perkin-Elmer GeneAmp 9700 system (Wellesley, MA).

Minisequencing. Five SNPs were analyzed simultaneously, three in the CYP1B1 gene, reported here, and one in the COMT gene and one in the CYP17 gene,
reported elsewhere. Because the two polymorphisms m1 and m2 are linked in Caucasians (13, 31), we analyzed only m2 because the corresponding fragment was easier to amplify in the multiplex PCR. SNPs were detected by specific extension with single fluorescein-labeled (NEN/ DuPont, Boston, MA) dideoxynucleotide triphosphates of a primer that anneals immediately adjacent to the variable site. To minimize pipetting steps, we used an AutoLoad kit (Amersham Pharmacia Biotech, Uppsala, Sweden), wherein PCR products were immobilized on streptavidin-coated comb-shaped manifold supports. The 25 μl multiplex PCR reaction contained 100 ng DNA, 20 mmol/l Tris-HCl (pH 8.4), 50 mmol/l KCl, 0.2 mmol/l of each deoxynucleotide triphosphate, 1.8 mmol/l MgCl₂, 10% DMSO, 0.625 units Platinum Taq polymerase (Life Technologies, Rockville, MD), and 0.25 μmol/l primer. The initial denaturation was done at 95°C for 2 minutes followed by 35 cycles each consisting of denaturation at 95°C for 15 seconds, annealing at 56°C, and extension at 72°C for 1 minute followed by a final extension at 72°C for 7 minutes. We checked that all fragments in the multiplex PCR reaction were amplified on agarose gels. Biotinylated PCR products were analyzed on an ALF DNA Autosequencer (Amersham Pharmacia Biotech). The extended primers were analyzed on an ALF DNA Autosequencer (Amersham Pharmacia Biotech) and the chromatograms were interpreted by direct visual inspection.

**DASH.** The PCR mix contained 10 ng DNA, 15 mmol/l Tris-HCl (pH 8.0), 50 mmol/l KCl, 0.12 μmol/l/biotinylated 5’ primer, 0.6 μmol/l 3’ primer, 0.2 μmol/l of each deoxynucleotide triphosphate (HPLC purified, Integrative GmbH, Ulm, Germany), 3.0 mmol/l MgCl₂, 5% DMSO, 0.6 units AmpliTaq Gold polymerase (Applied Biosystems, Foster City, CA) in a total volume of 25 μl. The initial denaturation was done at 95°C for 10 minutes followed by 38 cycles each consisting of denaturation at 95°C for 15 seconds and annealing at 60°C for 30 seconds followed by a final extension at 72°C for 4 minutes. PCR products were checked on 2% low-melting agarose gels. DASH assays were done as described by Prince et al. (30) and genotypes were scored from fluorescence curves as described by Howell et al. (32).

**Statistical Analyses.** We determined whether CYP1B1 genotype frequencies were in Hardy-Weinberg equilibrium using standard χ² statistics. We used the Expectation-Maximization algorithm to reconstruct haplotypes (33). Odds ratios (OR) and 95% confidence intervals (95% CI) were calculated from conditional logistic regression models using the maximum likelihood method. Models were conditioned on age group and on sampling category to ensure that age-associated nonparticipation or oversampling of long-term menopausal hormone users and diabetics did not introduce bias in the overall analyses. We also investigated whether the effect of CYP1B1 genotype varied over strata of menopausal hormone use (ever or never). Due to our sampling scheme, we could not estimate the joint effect of genotype and hormone use, but we studied the effects by genotype. Subgroups were defined by type of regimen: use of estrogen only (low-potency estrogens: estradiol 1-2 mg/d taken orally or medium-potency estrogens: conjugated estrogen, estradiol, and other synthetic estrogens) or use of medium-potency estrogens plus progestins taken continuously. Furthermore, subgroups were defined by duration of use (0, <2, or ≥2 years for medium-potency estrogens and 0, <5, or ≥5 years for low-potency estrogens). The duration periods were based on cutoff points wherein effects were seen in previous studies by Weiderpass et al. (24, 26). In secondary analyses, we explored possible interactions between CYP1B1 genotype and body mass index (BMI; <28 or ≥28 kg/m²), smoking (ever >100 cigarettes or never), combined oral contraceptives (ever or never), and family history. All analyses were done both on all cases (type 1 and type 2 tumors) and after selection of only endometrial cancer cases (type 1). To assess the potential for confounding, we introduced the variables listed in Table 1 into the logistic regression model. None of the

Table 1. Descriptive characteristics among CYP1B1 genotyped cases and controls

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>Cases/controls (n)</th>
<th>Cases</th>
<th>Controls</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age, y</td>
<td>689/1,549</td>
<td>63.9 ± 6.8</td>
<td>62.8 ± 6.6</td>
<td>&lt;0.0001*</td>
</tr>
<tr>
<td>Age at first term pregnancy, y</td>
<td>592/1,391</td>
<td>24.6 ± 4.5</td>
<td>24.8 ± 4.7</td>
<td>0.44</td>
</tr>
<tr>
<td>Age at last birth, y</td>
<td>592/1,391</td>
<td>29.5 ± 5.1</td>
<td>30.4 ± 5.3</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Age at menopause, y</td>
<td>601/1,490</td>
<td>51.1 ± 4.0</td>
<td>50.1 ± 4.0</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Parity (n children)</td>
<td>689/1,549</td>
<td>1.9 ± 1.2</td>
<td>2.1 ± 1.3</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Recent BMI, kg/m²</td>
<td>688/1,532</td>
<td>27.3 ± 5.3</td>
<td>25.5 ± 4.3</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Duration of menopausal hormone use, y</td>
<td>489/1,103</td>
<td>72.0</td>
<td>72.2</td>
<td></td>
</tr>
<tr>
<td>&lt;2</td>
<td>48/128</td>
<td>7.1</td>
<td>8.4</td>
<td></td>
</tr>
<tr>
<td>≥2</td>
<td>441/296</td>
<td>20.9</td>
<td>19.4</td>
<td>0.46</td>
</tr>
<tr>
<td>Ever use of oral contraceptives</td>
<td>165/544</td>
<td>24.1</td>
<td>35.4</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>Smoking</td>
<td>245/669</td>
<td>35.6</td>
<td>43.2</td>
<td>0.0007</td>
</tr>
<tr>
<td>First-degree family history of endometrial cancer</td>
<td>64/71</td>
<td>9.8</td>
<td>5.1</td>
<td>&lt;0.0001</td>
</tr>
</tbody>
</table>

*All analyses were adjusted for age.

*Recent refers to 1 year prior to data collection.

*Defined as a lifetime consumption of at least 100 cigarettes or regular smoking for >1 year.
variables altered the effect estimates of CYP1B1 genotype. Furthermore, we investigated if there was any association between genotype and the risk factors among controls by paired \( t \) test or by \( \chi^2 \) statistics but found no such covariations. All analyses were done using the SAS system PHREG, UNIVARIATE, or FREQ procedures (Release 8.01, SAS Institute, Inc. Cary, NC).

### Results

We successfully genotyped 689 endometrial cancer cases and 1,549 controls for the CYP1B1 m2, m3, and m4 SNPs. Genotype frequencies among controls were in Hardy-Weinberg equilibrium \( (P = 0.33 \text{ for } m2, P = 0.46 \text{ for } m3, \text{ and } P = 0.39 \text{ for } m4) \). We used the Expectation-Maximization algorithm (33) to reconstruct haplotypes from observed genotype data, as shown in Table 2. Estimated haplotype frequencies among controls were 0.086, 0.291, 0.452, and 0.169 for \( m3 \), and 0.33, 0.46, and 0.41 for \( m4 \). We investigated whether CYP1B1 genotype had an effect on endometrial cancer risk among women with a family history of endometrial cancer or among users of combined oral contraceptive but found no associations (data not shown). We also did overall and subgroup analyses after selection of only endometroid cancer cases (type 1), because these tumors are more estrogen dependent than type 2 tumors. The results obtained from these analyses (data not shown) did not differ from the results obtained from analyses on all endometrial cancer cases.

### Discussion

In this first population-based case-control study of CYP1B1 genotype and endometrial cancer risk, we found no overall association among Swedish postmenopausal women. Considering CYP1B1 as a generator of potentially genotoxic catechol estrogens, we found no evidence for our \( a \) priori hypothesis that an effect of CYP1B1 genotype would be more pronounced among those highly exposed to female sex hormones, such as users of menopausal estrogen or overweight women. The protective effect of smoking on endometrial cancer risk has been postulated to involve an antiestrogenic mechanism, whereby estrogen metabolism may be induced. Indeed, tobacco smoke–related compounds such as benzo(a)pyrene have been shown to induce enzymes involved in estrogen degradation in human endometrial cells (34) and in human liver (35). Induction of CYP1B1 among smokers could be of importance with regard to endometrial cancer risk; however, in this study, we found no support for such mechanism. Neither did we detect any association among oral contraceptive users. Overweight women carrying the CYP1B1*3 allele had a tendency for increased endometrial cancer risk; however, only the risk estimates for heterozygous were statistically significant, suggesting that this association was a chance finding.

In another study performed by us (14), wherein we investigated CYP1B1 genotype in association to breast cancer risk in the same population, we similarly found no overall association. Because the effect of CYP1B1...
genotype on estrogen-related cancer risk only may be manifested in conjunction with other exposures, we did hypothesis-based stratified analysis to investigate such interactions. Although numerous tests were done, we did not correct for multiple testing because all analysis were based on preconceptions about whether an association/interaction is biologically conceivable.

Strengths of this study include its population-based design, uniform histopathologic classification, and size. We had 85% statistical power to detect relative risks for endometrial cancer of $\geq 1.4$ to $\geq 1.9$ for the different CYP1B1 genotypes in overall analysis, given a 5% significance level. Furthermore, with detailed information on hormone regimens and other important risk factors, we could study possible gene-environment interactions; however, power in subgroups were limited.

Our study is not in accordance with the study on 113 Japanese cases and 202 Japanese controls done by Sasaki et al. (23), who found statistically significant associations between endometrial cancer risk and CYP1B1 genotype. The discrepancy may reflect that there are actually no differences between wild-type and variant alleles and that the Japanese finding is due to low sample size.

The evident link between endogenous or exogenous estrogen exposure and endometrial cancer makes this disease a suitable model system for studying the effect of genetic variation of estrogen metabolism. In this study, we found no evidence for such an effect of polymorphisms of CYP1B1.

Acknowledgments
We thank all participating women who made this study possible, Dr. Jonathan Prince for access to DASH apparatus, Dr. Anders Lindgren for pathologic review of all tumors, the primary health care centers and pathology departments all over Sweden for help with collecting blood and tissue samples, and Hans-Olov Adami and John Baron for invaluable advice since the inception of the project.

Table 4. Association between CYP1B1 genotype and endometrial cancer risk stratified by menopausal hormone use (type of regimen) with ORs and 95% CIs

<table>
<thead>
<tr>
<th>SNP</th>
<th>Genotype</th>
<th>Low-potency estrogen</th>
<th>Medium-potency estrogen</th>
<th>Medium-potency estrogen plus progestins continuously</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Never</td>
<td>Ever</td>
<td>Never</td>
</tr>
<tr>
<td></td>
<td>OR*</td>
<td>95% CI</td>
<td>OR*</td>
<td>95% CI</td>
</tr>
<tr>
<td>m2</td>
<td>355G/G</td>
<td>187/470</td>
<td>1</td>
<td>62/67</td>
</tr>
<tr>
<td></td>
<td>355G/T</td>
<td>176/354</td>
<td>1.2</td>
<td>1.0–1.6</td>
</tr>
<tr>
<td>m3</td>
<td>432G/C</td>
<td>109/260</td>
<td>1</td>
<td>44/52</td>
</tr>
<tr>
<td></td>
<td>432G/C</td>
<td>203/450</td>
<td>1.1</td>
<td>0.8–1.4</td>
</tr>
<tr>
<td>m4</td>
<td>4390A/A</td>
<td>280/609</td>
<td>1</td>
<td>90/103</td>
</tr>
<tr>
<td></td>
<td>4390A/G + 4390G/G</td>
<td>113/291</td>
<td>0.9</td>
<td>0.7–1.1</td>
</tr>
</tbody>
</table>

The logistic regression models were conditioned on age in 5-year intervals, duration of use of menopausal estrogen or estrogen plus progestins, and diabetes mellitus.

Table 5. Association between CYP1B1 genotype and endometrial cancer risk stratified by BMI with ORs and 95% CIs

<table>
<thead>
<tr>
<th>SNP</th>
<th>Genotype</th>
<th>BMI &lt; 28</th>
<th>BMI ≥ 28</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Cases/controls (n)</td>
<td>OR*</td>
</tr>
<tr>
<td>m2</td>
<td>355G/G</td>
<td>191/555</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>355G/T</td>
<td>176/432</td>
<td>1.1</td>
</tr>
<tr>
<td>m3</td>
<td>432G/C</td>
<td>123/325</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>432G/G</td>
<td>203/544</td>
<td>0.9</td>
</tr>
<tr>
<td>m4</td>
<td>4390A/A</td>
<td>288/726</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>4390A/G + 4390G/G</td>
<td>117/358</td>
<td>0.9</td>
</tr>
</tbody>
</table>

The logistic regression models were conditioned on age in 5-year intervals, duration of use of menopausal estrogen or estrogen plus progestins, and diabetes mellitus.
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References

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