Estrogen Bioactivation, Genetic Polymorphisms, and Ovarian Cancer

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Abstract

Recent experimental evidence has shown that catechol estrogens can be activated through metabolism to form depurinating DNA adducts and thereby initiate cancer. Limited data are available regarding this pathway in epithelial ovarian cancer. We conducted a case-control study of 503 incident epithelial ovarian cancer cases at the Mayo Clinic in Rochester, MN, and Jacksonville, FL, and a 48-county region in North Carolina. Six hundred nine cancer-free controls were frequency matched to the cases on age, race, and residence. After an interview to obtain data on risk factors, a sample of blood was collected for DNA isolation. Subjects were genotyped for seven common single nucleotide polymorphisms in four genes involved in catechol estrogen formation (*CYP1A1* and *CYP1B1*) or conjugation (*COMT* and *SULT1A1*). Data were analyzed using logistic regression, stratified by race, and with adjustment for design factors and potential confounders. None of the individual genotypes were significantly associated with ovarian cancer risk. However, an oligogenic model that considered the joint effects of the four candidate genes provided evidence for an association between combinations of these genes and ovarian cancer status ($P = 0.015$). Although preliminary, this study provides some support for the hypothesis that low-penetrance susceptibility alleles may influence risk of epithelial ovarian cancer. (Cancer Epidemiol Biomarkers Prev 2005;14(11):2536–43)

Introduction

Among the cancers specific to women, ovarian cancer has the highest mortality rate. Each year in the United States, ~26,000 women are diagnosed with ovarian cancer and 14,000 die of it. Despite the public health importance of ovarian cancer, little is understood about its etiology. Nulliparous women are at a higher risk than parous women, and each additional pregnancy lowers risk by ~15% (1, 2). Other aspects of reproductive history asso-ciated with increased risk for breast cancer (age at menarche, age at menopause, and age at first birth) are not clearly associated with ovarian cancer risk. Obesity is not a consistent risk factor, although some data suggest that body fat distribution (in particular, abdominal adiposity) is a risk factor (3).

An extensive review of the hormonal etiology of epithelial ovarian cancer (4) concluded that there are two, not necessarily mutually exclusive, hypotheses that reflect what is currently known about the disease. The first hypothesis states that ''incessant ovulation'' causes cancer through repeated disruption of the ovarian surface epithelium and formation of stromal epithelial clefts and inclusion cysts. The second hypothesis, often called the gonadotropin hypothesis, posits that some type of hormonal stimulation of ovarian epithelial cells, either on the surface of the ovary or within ovarian inclusion cysts, is the relevant pathway. Moreover, several lines of evidence suggest the importance of estrogens as the relevant hormone. For instance, estrogen receptors have been found in cytosols of normal and benign ovaries (5-9) and other studies have confirmed the expression of both estrogen receptor α and β in human ovarian corpus luteum tissue (10) and cultured ovarian surface epithelial cells (11). As well, increased estrogenic influences during the menstrual cycle have been shown to increase the proliferation of the epithelium, whereas reports on hormone replacement therapy (HRT) with conjugated estrogens have also indicated increased risk of ovarian cancer. Conversely, tubal ligation and use oral contraceptives seem to lower risk (12).

There is equally strong evidence to suggest that androgens or progesterone play a role in the etiology of ovarian cancer (4). For example, the protective effect of pregnancy may be ascribed to the 100-fold increases in serum progesterone levels (13). In addition, plasma concentrations of androgens—even during the late follicular phase of the menstrual cycle when estrogens are at their peak—are greater than estrogens (14). It has also been shown that postmenopausal ovaries are particularly androgenic, with concentrations of testosterone ~15-fold higher in ovarian vein serum than peripheral vein serum (15). Finally, androgen receptors have frequently been found in normal ovaries and have been identified within ovarian epithelial cells (16).

The lack of certainty about the hormonal etiology of ovarian cancer may reflect the inadequacy of the current paradigm that hormones influence the risk of epithelial cancers through receptor-mediated pathways. Recent experimental evidence has shown that a non-receptor-mediated pathway may play a role in the initiation of cancer. In this pathway, catechol estrogens are oxidized to activated species that react with DNA to form depurinating adducts and thereby initiate cancer (17, 18). Cytochrome *P*450 CYP1A1 and CYP1B1 catalyze the hydroxylation of estrogens to form the catechol estrogens (19). The gene for CYP1A1 contains a common polymorphism

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(*A4889G*) that encodes amino acid *Ile*^{462}*Val* in the heme-binding region of the protein (20). When expressed in a yeast expression system, the less common *Val1*^{462} variant is reported to show a 2-fold increase in ability to catalyze the oxidation of benzo[*a*]pyrene (21). The *CYP1B1* gene has four common polymorphisms with the open reading frame that alter the encoded amino acid (22): a C-to-T transition that encodes amino acid *Arg*^{48}*Gly*, a G-to-T transversion that encodes amino acid Ala^{119}Ser, a C-to-G transversion that encodes the amino acid Leu^{432}Val and finally an A-to-G transition that encodes the amino acid Asn^{453}Ser (22).

Catechol-*O*-methyltransferase (COMT) catalyzes catechol estrogens to form methyl conjugates, a process that detoxifies the catechol estrogens and prevent them from forming depurinating adducts. COMT exists as both cytosolic and membrane-bound forms transcribed from a single gene with two different sites of transcription initiation (24). A polymorphic G-to-A transition in COMT encodes the amino acid Val$^{108/158}$Met (25), which displays low levels of both enzyme activity and immunoreactive protein in every human tissue that has been studied (26). In addition to methyl conjugation, sulfate conjugation catalyzed by sulfotransferases inactivates the catechol estrogens (27), and SULT1A1, a ubiquitously expressed sulfotransferase isoform that catalyzes the catechol estrogen, has one common polymorphism (G638A) that encodes the amino acid change Arg^{213}His. Subjects homozygous for the variant allele (His^{213}) express low levels of SULT1A1 enzyme activity (28).

Limited data are available regarding this pathway in epithelial ovarian cancer. The purpose of the current study was to examine the association of functional genetic polymorphisms of genes involved in the oxidative metabolism of estrogens to form catechol estrogens (*CYP1A1* and *CYP1B1*) or conjugation of catechol estrogens through methylation (*COMT*) and sulfation (*SULT1A1*) in a large, multicenter case-control study of ovarian cancer.

Materials and Methods

Patient Population. The patients recruited for this research project were identified through two institutions: Duke University and Mayo Clinic. The protocol was approved by the institutional review board at each institution, and all study subjects provided signed informed consent. Although there were several differences in the specific study designs used at each site, the overall inclusion-exclusion criteria were similar. To be eligible for the study, cases must have been ascertained within 1 year of a diagnosis of histologically confirmed primary epithelial ovarian cancer. Women with a history of ovarian cancer were ineligible. We included Caucasian and African American women with either borderline or invasive tumors but excluded those with sarcoma, germ-cell tumors, or sex-cord stromal tumors. The lower age limit was 20 years at both sites, but Mayo had no upper age limit, whereas Duke excluded women ages >74 years.

The Mayo ascertainment was hospital based and began in January 2000 in Rochester, MN, and January 2001 in Jacksonville, FL. The catchment area for Mayo Rochester was limited to the six-state region that represents >85% of all ovarian cancer cases seen there: Minnesota, Iowa, Wisconsin, Illinois, North Dakota, and South Dakota. Similarly, ascertainment at Mayo Jacksonville was limited to Florida, Georgia, and South Carolina, as 87% of ovarian cancer cases seen there are from these states. Although Mayo is widely perceived to be a specialty tertiary care facility, it provides primary care for many women as well. Because a basic tenet of case-control studies is that cases and controls should be selected from the same source population (29), we selected clinic-based controls from healthy women seeking general medical examination and frequency matched to cases on age (5-year age category), race, and state of residence. Potential controls were excluded if they had a prior oophorectomy. Response rates for those invited to participate at the Mayo site was 89% for cases and 80% for controls.

The Duke study is population based with a rapid case ascertainment network covering a 48-county region of North Carolina. Recruitment has been ongoing since January 1, 1999. List-assisted random digit dialing and Health Care Financing Administration roster methods were used to identify control subjects. Controls were frequency matched to the cases based on race (Black versus non-Black) and age (5-year age categories). The response rate among eligible cases was 76%. Nonresponders were classified as patient refusal (7%), inability to locate the patient (8.5%), physician refusal (3%), death (4%), or debilitating illness (2%). The response rate for the study was 65% among the controls.

Risk Factor Data Collection. Information on known and suspected ovarian cancer risk factors and demographic data were collected through in-person interviews. Similar questionnaires were used at each institution. Information collected included race/ethnicity, menstrual and reproductive history, use of exogenous hormones, medical and surgical history, height and weight 1 year before the interview, use of tobacco, education level, and family history of breast or ovarian cancer in first-degree relatives.

Collection and Processing of Biospecimens. Genomic DNA was obtained from cases and controls in one of two ways. For the Duke protocol, venipuncture was done at the conclusion of the interview. At Mayo, participants had an extra vial of blood drawn during their scheduled medical care.

Genomic DNA was isolated from 10 to 15 mL whole blood, resuspended in TE buffer, and stored at 4°C. The DNA concentration was determined by UV spectroscopy and diluted to 5 ng/μL before genotyping.

Genotyping. The *CYP1A1, CYP1B1,* and *COMT* single nucleotide polymorphisms (SNP) were analyzed using a chip-based platform (Nanogen, San Diego, CA). A description of the methodology and its application has been described previously (30). The primer sequences for PCR, probe and stabilizer, and annealing temperature are available on request. Briefly, genomic DNA (50-20 ng) was PCR amplified with the specific primers, each at 1.0 μmol/L, 400 μmol/L deoxynucleotide triphosphates, 1.5 mmol/L $MgCl_2$, 0.6 units Qiagen (Valencia, CA) Taq and 3.0 μL Qiagen Q solution in a 15-μL reaction. Each PCR reaction was as follows: 12 minutes at 95°C followed by 35 cycles at 94°C for 30 seconds, annealing temperature for 30 seconds, and 72°C for 30 seconds, with a final extension at 72°C for 10 minutes.

Biotinylated amplicons were desalted using Millipore Multiscreen PCR plates (Billerica, MA), transferred to Nunc (Rochester, NY) V-bottomed plates, and resuspended in 50 mmol/L L-histidine to 5 to 40 nmol/L. Amplicon and L-histidine were electronically addressed by the Nanogen software to the designated sites. Following denaturation with 0.1 mol/L NaOH, hybridization mixture (250 mmol/L of the stabilizer oligonucleotide and 500 mmol/L of the reporter probe nucleotides) was added (5 minutes in the dark). Each microarray was imaged with separate lasers (for Cy3 and Cy5) and a temperature was selected to discriminate between matched and mismatched reporters. Known heterozygotes were used to normalize hybridization efficiency between dye-labeled reporters. Genotypes were determined by biallelic fluorescence intensity ratios: ≥1:30 was deemed heterozygous and ≤1:5 was deemed homozygous.

SULT1A1 genotyping was carried out by pyrosequencing on a PSQ96 system (Pyrosequencing, Foxboro, MA). Specifically, the PCR reaction contained 25 ng template DNA, 16.7 pmol of each primer (5′-bioTEG-GTTGGCTCTGCAGGGTCTCTAGGA-3′

and 5′-GTGTGCTGAACCATGAAGTCCACG-3′), 200 μmol/L deoxynucleotide triphosphates, 2.0 mmol/L $MgCl_2$, and 1.0 units AmpliTaq Gold (Applied Biosystems, Foster City, CA) in a 25-μL reaction. The cycling variable consisted of 12 minutes at 95°C followed by 35 cycles at 94°C for 30 seconds, 63.5°C annealing temperature for 30 seconds, and 72°C for 30 seconds, with a final extension at 72°C for 10 minutes. PCR cleanup was carried out according to Pyrosequencing Vacuum Prep protocol. PCR products were mixed with 15 μL H_2O, 37 μL binding buffer, and 3 μL streptavidin-Sepharose beads. Mixture was incubated/agitated for 5 minutes at room temperature. Using the Vacuum Prep workstation, beads were transferred to 70% ethanol (10 seconds), to 0.2 mol/L NaOH (10 seconds), and to 1× washing buffer (10 seconds) and then released into 40 μL of 1× annealing buffer containing 0.4 μmol/L annealing primer (5′-CGGTCTCCTCTGGCA-3′). After denaturation at 80°C for 3 minutes, samples were then subjected to DNA sequencing on a PSQ96 system. The genotype of each sample was called automatically by the instrument but also evaluated manually for potential genotype misclassification. Two Centre d'Etude du Polymorphisme Humain controls, each in duplicate and two no-template wells, are included in each 96-well plate to control for genotype call and contamination.

Statistical Analysis. Before analysis, we determined descriptive statistics using frequencies and percents for categorical variables and means and SDs for continuous variables. The distributions of covariates were compared across study site and case status using ANOVA methods for continuous variables and χ^2 tests for categorical variables. SNP genotype frequencies among the controls were tested for Hardy-Weinberg equilibrium (HWE) using χ^2 goodness-of-fit tests.

Unconditional logistic regression was used to estimate odds ratios (OR) and corresponding 95% confidence intervals (95% CI) between SNP genotypes and case status. Due to differential racial genotype frequencies, separate logistic regression models were fit for Caucasian and African American subjects; individuals self-reported as being of other races were excluded. Individuals homozygous for the wild-type (WT) allele were designated the reference category. For Caucasian subjects, a 2-*df* test was used whenever possible to assess differences in genotypes present in the cases versus the controls. For one SNP, the minor allele frequency was so low that the heterozygous and homozygous variant genotypes were combined. Smaller sample sizes for the African American subjects required us to combine heterozygous genotypes with one of the two homozygous genotypes. For most SNPs, we combined the heterozygotes and the homozygous variants. However, the low frequency of the WT *CYP1B1/L432V* SNP in the African Americans required us to combine the heterozygotes with the homozygous WT genotypes.

SNP associations were initially assessed separately for the Mayo and Duke sites. We assessed the potential modifying effects of study site on SNP associations by statistically testing the interaction between site and each of the SNPs of interest. If we found no evidence of effect modification, we pooled data from both sites.

All models were adjusted for the design variables of study site and age. To assess the possibility that other demographic and behavioral variables would confound the association between the SNPs of interest and ovarian cancer, we ran a series of formal confounding analyses on the following set of predetermined potentially confounding covariates using a ''change-in-estimate'' approach (31): education, age at menarche, number of live births, age at first live birth, fertility problems, oral contraceptive use, menopausal status, HRT use, family history of breast or ovarian cancer, smoking status, and body mass index.

In addition to performing single-SNP analyses, we assessed the potential modifying effects of certain demographic and clinical variables by fitting a series of gene × environment interaction models. Analyses were restricted to Caucasian American subjects, as the number of African American subjects in this study was insufficient to support such models. To further avoid the possibility of sparse table cells, each SNP was modeled as a dichotomous variable based on the presence (one or two copies) or absence (zero copies) of the variant allele. Similarly, each environmental variable was dichotomized based on either a median split of the data or a pooling of categories. The modifying effects of the following variables were considered: ever smoked, body mass index (median split), live births (0 versus ≥1), ever used oral contraceptives, ever used hormone replacements, and menopausal status. Combinations of these six environmental variables with the six SNPs resulted in a total of 36 assessments of interaction. For each interaction, two models were fit. First, we included the main effects of the corresponding pair of variables along with their interaction. Statistical significance of the interaction was assessed using a 1-*df* Wald's test. To better interpret the information, we then fit a model that included all but one of the four combinations of the two variables. The one combination excluded from the model (hypothesized *a priori* to have the lowest risk of ovarian cancer) served as the reference group for this comparison.

We next examined the possibility that combinations of the SNPs might be associated with ovarian cancer status. These oligogenic analyses were also restricted to Caucasian American subjects due to the relatively low number of enrolled African American subjects. Even in the Caucasian American subjects, the number of potential combinations of alleles was prohibitively large when performing analyses on all seven SNPs. We therefore assessed pair-wise linkage disequilibrium of the four *CYP1B1* SNPs using the *D′* statistic (32). Following the method outlined by Carlson et al. (33), we formed bins of SNPs where all values of pair-wise *D′* were >0.95 and selected a single-tagging SNP from each bin. We selected the SNP with the highest minor allele frequency within the bin as the tag SNP.

To evaluate the potential for oligogenic correlates of ovarian cancer status, we did analyses that examined associations between combinations of the alleles at a single SNP from each of the four genes of interest. Our analytic approach was similar to the way in which haplotype analyses are done using unphased genotypes (see refs. 34, 35). That is, we created variables that represented specific combinations of unphased alleles, ''carrier combinations'' rather than haplotypes. To achieve these oligogenic analyses, we created a variable for each of the 2^4 = 16 possible allele combinations from the four SNPs under consideration. For each subject, the value of the variable corresponding to a specific combination of SNP variants was set equal to the number of copies of that particular combination that was consistent with the genotypes of the individual. For instance, if a subject was homozygous for the WT allele for all four of the SNPs, then the value of the variable representing the WT-WT-WT-WT combination was set equal to 2 and the value of each of the other carrier combination variables was set equal to 0, and subjects who were heterozygous for all SNPs were coded as carrying one copy of all possible carrier combinations. We used all of these coded variables in a single logistic regression model and tested the association between the observed carrier combinations and ovarian cancer status using the resulting multiple degree of freedom likelihood ratio χ^2 statistic. In addition to testing the global null hypothesis of there being no association between these oligogenic effects and ovarian cancer status, we also estimated ORs representing the relative difference in ovarian cancer risk associated with carrying an additional copy of each multiallelic combination while controlling for all other carrier combinations.

To further simplify the interpretation of the oligogenic model, we also grouped the genotype carrier combinations into five classes based on the probability of being an ovarian

cancer case as predicted by the model. We selected the groups such that there were approximately equal numbers of subjects per grouping. We then obtained estimates of the ORs for case status within each of these five groups.

Results

The current report is based on a total of 503 incident cases (197 at Mayo and 306 at Duke) and 609 controls (210 at Mayo and 399 at Duke). The clinical and pathologic characteristics of the cases are shown in Table 1. The proportion of borderline tumors was similar at both sites. There was a statistically significant difference in the overall distribution of histologic subtypes of the tumors, with slightly more serous and endometrioid tumors at Mayo and more mucinous and other histologies at Duke. Mayo had a slightly greater proportion of stage IV cases, whereas Duke had a slightly greater proportion of stage I cases. The distribution of grade tended to be higher at Mayo than Duke.

The distribution of nongenetic risk factors by study site is shown in Table 2. Because participants were matched on age and race, there were no differences between cases and controls on these variables. Because of different eligibility on age, however, the Mayo cases and controls tended to be an average of 5 years older than the corresponding cases and controls at Duke ($P < 0.01$). Cases were less educated than controls, but this difference was evident only among the Mayo subjects. Cases had earlier age at menarche and were more likely to be nulliparous and report difficulty in becoming pregnant. Risk of ovarian cancer was inversely associated with use of oral contraceptives; however, this difference was statistically significant only at Mayo ($P = 0.02$) and not at Duke ($P = 0.09$). Cases had a higher body mass index and HRT use than controls, but there were no differences between cases and controls in family history of breast or ovarian cancer or cigarette smoking (status or duration).

Before examining the association of the candidate gene polymorphisms with ovarian cancer risk, we confirmed that genotypes for each were consistent with HWE. We found that the Caucasian control subjects from Duke presented fewer heterozygotes for the *CYP1B1-453* SNP than would be expected under HWE. We visually reexamined the data for every genotyping run and the assays were very clean. In addition, the 432 SNP is on the same exon, and for this analysis, it was coamplified with 453. Thus, there were no obvious genotyping errors. To explore further, we reanalyzed for the *N453S* variant using a different genotyping platform (SNPstream). The genotypes were still not in HWE. We therefore excluded this SNP from further analysis. We also compared genotype frequencies by race and study site. African American and Caucasian subjects had significantly different allele or genotype frequencies for *COMT* and all four *CYP1B1* SNPs. Thus, all subsequent analyses were stratified by race. Stratification by study center revealed no evidence of site-specific effect modification; hence, all other associations used pooled data from Mayo and Duke. Multivariate models were fit to evaluate the potential confounding effects of the nongenetic risk factors listed in Table 2, but none influenced the point estimates of effect by ≥10%. Therefore, we opted for the most parsimonious models, which included adjustment only for age and study center. ORs and 95% CIs for the genotypes of interest by self-reported race are provided in Table 3. None of the gene polymorphisms involved in catechol estrogen formation (*CYP1A1* and *CYP1B1*) or conjugation (*COMT* and *SULT1A1*) were individually associated with risk among Caucasian cases and controls. Among the few African American subjects, no significant associations were evident. Results did not differ after excluding the borderline cases.

Table 1. Clinical and pathologic description of epithelial ovarian cancer cases by study site

Variable	Level	Mayo (n = 197)	Duke (n = 306)	P
Invasion	Borderline	35 (17.8)	70 (22.9)	0.17
	Invasive	162 (82.2)	236 (77.1)	
Histology	Serous	131 (66.5)	182 (59.5)	0.05
	Mucinous	16 (8.1)	40 (13.1)	
	Endometrioid	32 (16.2)	37 (12.1)	
	Clear cell	8 (4.1)	15 (4.9)	
	Other	10 (5.1)	32 (10.4)	
Stage	Missing	16	1	0.04
	I	46 (25.4)	105 (34.4)	
	II	14 (7.7)	22 (7.2)	
	III	99 (54.7)	160 (52.5)	
	IV	22 (12.2)	18 (5.9)	
Grade	Missing	10	23	<0.01
	Borderline	35 (18.7)	70 (24.7)	
	1	4 (2.1)	32 (11.3)	
	2	18 (9.6)	71 (25.1)	
	3	77 (41.2)	101 (35.7)	
	4	53 (28.3)	9 (3.2)	

NOTE: n (%) and Ps are from χ^2 test of significance.

The next set of analyses was designed to test the hypothesis that the high-risk alleles may have a stronger association among strata of risk factors associated with exposure to hormones. This included factors that would increase hormone exposures (body mass index, nulliparity, HRT use, and premenopausal status) as well as those that would decrease exposure (smoking status and oral contraceptive use). Statistically significant interactions were detected between nulliparity ($P = 0.05$), HRT use ($P < 0.05$), and *CYP1B1* (*R48G* and *A119S*) and between smoking status ($P < 0.01$), menopause status ($P < 0.05$), and *CYP1A1* (*V462I*). Compared with the reference group of nonsmokers with no copies of the high-risk allele, carriers of the *CYP1A1* variant were at decreased risk if they smoked (OR, 0.29; 95% CI, 0.13-0.64) but not if they never smoked (OR, 1.62; 95% CI, 0.84-3.14). The interaction between *CYP1A1* and menopause status was driven by the observation that premenopausal carriers of the *CYP1A1* variant were at decreased risk relative to the other three genotype/menopause categories. This effect was seemingly stronger in the Mayo subjects than the Duke subjects, although associations did not statistically differ by study site. When we examined this association by study site, however, the direction of the association differed (data not shown). The interactions between *CYP1B1* and parity were consistent for both variants and suggest that nulliparous noncarriers were at increased risk relative to carriers or parous noncarriers (data not shown). The two *CYP1B1* variant interactions with HRT use were also consistent, suggesting that the *R48G* carriers who were exposed to HRT were at 2.0-fold (95% CI, 1.45-3.04) elevated risk relative to noncarriers/nonusers and that *A119S* carriers who were exposed to HRT were at 2.0-fold greater risk (95% CI, 1.40-2.94). This finding was driven primarily by results among the Duke subjects.

Although the individual SNPs were each only weakly associated with the risk of epithelial ovarian cancer, we reasoned that it might be instructive to construct a multigene model that captured variation across the pathway. Because of the few African American subjects and the racial differences in allele frequency, this analysis was restricted to the Caucasian subjects. In addition, the three valid SNPs within the *CYP1B1* gene were in tight linkage disequilibrium, with pair-wise D' statistics ranging from 0.97 to 1.0. We therefore selected *CYP1B1-432* to serve as the *CYP1B1* tag SNP, as it had the highest minor allele frequency. Thus, the final oligogenic model considered only four SNPs, one from each of the candidate genes. This oligogenic model provides some evidence that the SNPs from these four genes are simultaneously associated with ovarian cancer ($P = 0.015$). As shown

Table 2. Distribution of nongenetic risk factors for epithelial ovarian cancer by study site

Variable	Mayo		Duke		P^*
	Cases	Controls	Cases	Controls	
Mean age (y)	60.2 (13.3)	60.1 (13.0)	54.3 (11.3)	54.9 (13.0)	0.86
Race (% Caucasian)	195 (99.0)	208 (99.0)	272 (88.9)	347 (87.0)	0.30
Education (more than high school)	93 (51.1)	145 (69.0)	181 (59.2)	242 (60.7)	0.01
Mean age at menarche (y)	12.7 (1.3)	13.0 (1.6)	12.4 (1.6)	12.6 (1.4)	0.01
No. children					
0	34 (17.4)	30 (14.3)	57 (18.6)	54 (13.5)	0.08
1-2	71 (36.4)	69 (32.9)	162 (52.9)	211 (52.9)	
≥3	90 (46.2)	111 (52.9)	87 (28.4)	134 (33.6)	
Mean age at first birth (y)	23.1 (4.6)	23.6 (4.1)	22.6 (4.6)	23.3 (5.0)	0.05
Fertility problems	43 (22.1)	27 (13.1)	99 (32.4)	84 (21.1)	<0.01
Oral contraceptive use					
Never	91 (48.1)	86 (41.5)	103 (33.8)	134 (33.8)	<0.01
1-48 mo	50 (26.5)	42 (20.3)	101 (33.1)	104 (26.3)	
>48 mo	48 (25.4)	79 (38.2)	101 (33.1)	158 (39.9)	
Menopausal status (% postmenopausal)	131 (71.6)	156 (74.3)	211 (75.1)	257 (65.9)	0.08
HRT use					
Never	113 (59.5)	109 (55.9)	116 (38.5)	247 (63.5)	<0.01
1-60 mo	35 (18.4)	36 (18.5)	119 (39.5)	77 (19.8)	
>60 mo	42 (22.1)	50 (25.6)	66 (21.9)	65 (16.7)	
Family history of ovarian cancer	10 (5.1)	6 (2.9)	9 (2.9)	11 (2.8)	0.35
Family history of breast or ovarian cancer	44 (22.6)	34 (16.2)	48 (15.7)	64 (16.0)	0.32
Ever smoked (%)	66 (34.4)	78 (37.3)	144 (47.1)	200 (50.1)	0.24
Mean pack-years smoked	5.3 (14.6)	6.5 (16.7)	9.3 (15.5)	9.6 (15.9)	0.41
Mean body mass index (kg/m^2)	28.1 (6.5)	26.8 (5.9)	28.5 (7.4)	27.3 (6.4)	<0.01

NOTE: n (%) for categorical variables and mean (SD) for continuous variables. Variable-specific counts may not add up to overall counts due to missing values.
*Ps based on χ^2 test for categorical variables and Student's t test for continuous variables, comparing cases with controls and pooling together the data from the two study sites.

in Table 4, there was a wide variation in the magnitude of association of the various multivariate-adjusted allele combinations with risk. Contrary to our *a priori* expectations, however, the ORs did not increase with increasing number of variant alleles.

The complexity of the results from Table 4 prompted *post hoc* exploratory analyses in which we allowed the data to inform the groupings of genotypes. Thus, we grouped the genotype combinations into five classes, with approximately equal numbers of subjects according to the probability of being an ovarian cancer case that was predicted by the full oligenic model. This simplified model did not suffer from significant lack of fit when compared with the full oligogenic model (P = 0.389) while retaining the gradation of ovarian cancer risk across the genotype combination groupings. The point and interval estimates of these model-derived groupings are illustrated in Table 5 along with the SNP genotype combinations that comprise the different risk groups.

Discussion

The current study found that the allele and genotype frequencies of *COMT* and *CYP1B1* varied significantly by self-reported race. Race-specific analyses, however, provided no evidence for a strong association of any of the candidate gene polymorphisms individually with risk of ovarian cancer. Postulating that the combination of low-penetrance susceptibility

Table 3. Association of CYP1A1, CYP1B1, COMT, and SULT1A1 genotypes with risk of ovarian cancer stratified by race

Gene	No. alleles	Caucasian subjects			African American subjects		
		Cases	Controls	OR (95% CI)*	Cases	Controls	OR (95% CI)†
CYP1A1/V462I	0	423	496	1.0 (reference)	33	50	1.0 (reference)
	1/2	31	48	0.7 (0.5-1.2)	3	3	2.1 (0.4-12.5)
CYP1B1/R48G	0	235	290	1.0 (reference)	6	12	1.0 (reference)
	1	179	206	1.1 (0.8-1.4)	30	41	1.5 (0.5-4.6)
	2	39	47	1.0 (0.6-1.6)			
CYP1B1/A119S	0	233	285	1.0 (reference)	6	16	1.0 (reference)
	1	178	208	1.0 (0.8-1.4)	30	37	2.2 (0.7-6.6)
	2	42	50	1.0 (0.6-1.6)			
CYP1B1/L432V	0	140	166	1.0 (reference)	13	22	1.0 (reference)
	1	230	269	1.0 (0.8-1.4)			
	2	84	110	0.9 (0.6-1.3)	23	31	1.3 (0.5-3.5)
COMT/V158M	0	110	127	1.0 (reference)	19	23	1.0 (reference)
	1	224	269	1.0 (0.7-1.3)	17	30	0.8 (0.3-1.8)
	2	119	147	0.9 (0.7-1.3)			
SULT1A1/R213H	0	197	236	1.0 (reference)	15	25	1.0 (reference)
	1	194	237	1.0 (0.8-1.3)	21	28	1.3 (0.5-3.3)
	2	63	69	1.1 (0.8-1.7)			

*ORs (95% CIs) adjusted for age and study site.
†Due to small sample sizes, most SNP associations for the African American subjects combined heterozygous and homozygous variant genotypes into one group. The low frequency of the WT CYP1B1/L432V SNP in the African Americans required us to combine the heterozygous and homozygous WT genotypes into one group.

Table 4. Multigene model of estrogen bioactivation, SNPs, and ovarian cancer risk among Caucasian subjects

CYP1A1	CYP1B1/L432V	COMT/V158M	SULT1A1/R213H	Genotype combination frequency*	OR† (95% CI)
WT	WT	WT	WT	0.157	1.23 (0.94-1.6)
			Variant	0.101	0.97 (0.70-1.34)
		Variant	WT	0.008	0.93 (0.71-1.2)
			Variant	0.003	0.96 (0.68-1.4)
	Variant	WT	WT	0.182	1.02 (0.75-1.4)
			Variant	0.093	0.77 (0.51-1.2)
		Variant	WT	0.009	0.84 (0.63-1.1)
			Variant	0.003	1.51 (1.01-2.3)
Variant	WT	WT	WT	0.138	0.03 (0.00-0.35)
			Variant	0.070	8.11 (0.29-224.5)
		Variant	WT	0.006	45.16 (3.60-566.6)
			Variant	0.002	0.13 (0.00-4.5)
	Variant	WT	WT	0.145	6.26 (0.71-55.4)
			Variant	0.074	0.59 (0.02-15.1)
		Variant	WT	0.007	0.06 (0.01-0.76)
			Variant	0.003	1.07 (0.03-36.95)

*Frequencies estimated based on an enumeration of all possible genotype combinations given the observed single-SNP genotypes and applying equal weight to each of those genotypes within a subject such that the intrasubject weights sum to 1. The reported frequencies reflect the sum of these weights across all subjects.
†Estimated increase in odds of ovarian cancer for each additional copy of the genotype combination of interest after accounting for the effects of all other genotype combinations.

genes may lead to clinically important elevations in risk, we constructed an oligogenic model to look at the combined effects of the four candidate genes within the pathway. These results suggest that this line of research is likely to be fruitful. This model provided support for the presence of a potential association between combinations of these SNPs and ovarian cancer. However, no more than 9% of the variability in risk can be accounted for by these four candidate genes.

Although the literature on ovarian cancer and genetic variation is still quite limited, with no published studies of *SULT1A1* and ovarian cancer, the results from our study are in contrast to some previous studies that were based on smaller sample sizes. Four studies have reported on *CYP1A1* in relation to risk of ovarian cancer. Aktas et al. (36) studied 117 ovarian cancer patients and 202 controls in Turkey and observed that the *CYP1A1 Ile/Val* (heterozygous) genotype was associated with a 5.7-fold increased risk (95% CI, 3.3-9.8) and the *Val/Val* (homozygous) genotype was associated with even greater risk (OR, 7.4; 95% CI, 1.8-19.6). Increased risk for women carrying any *Val* allele was also seen for borderline tumors (OR, 4.6; 95% CI, 1.6-13.1) and for benign ovarian tumors (OR, 5.7; 95% CI, 1.6-13.1). Terry et al. (37) compared allele frequencies of the *Msp1* and *Ile/Val CYP1A1* variants in 445 ovarian cancer cases and 472 controls in New England and found no difference. However, women who possessed an *Ile/Val* variant and who consumed more than the median amount of animal fat daily were at a higher risk of ovarian cancer (relative risk, 2.2; 95% CI, 1.1-4.6) as were women who consumed more than median levels of caffeine daily (relative risk, 2.7; 95% CI, 1.2-6.2). There was no increased risk for women carrying the *Msp1* variant. Goodman et al. (38) studied 129 cases and 144 controls and found that women with at least one *Msp1* variant allele who smoked cigarettes were at 2.6-fold increased risk (95% CI, 1.2-6.0) compared with never-smoking women with the WT genotypes. A case-control study in Japan (39) found no significant differences between the frequency of either the *Ile/Val* allele or the *Msp1* allele. The current study observed no main effect of the *V462I* variant with risk, and the interaction with smoking was opposite to that observed by Goodman et al. (38).

CYP1B1 has at least seven common polymorphisms, but based on transfection experiments, only the *N453S* allele is functionally significant (40). Two previous studies have examined the *L432V* variant allele in *CYP1B1* in relation to ovarian cancer risk. In 2001, Goodman et al. (38) noted that compared with women with a *Leu/Leu* genotype those with *Val/Leu* genotype had an increased risk of ovarian cancer (OR, 1.8; 95% CI, 1.0-3.3) as did those with the *Val/Val* genotype (OR, 3.8; 95% CI, 1.2-11.4). Risks were increased for both Asian and Caucasian subjects and were higher among smokers, nulliparous women, and never users of oral contraceptives. In contrast, Cecchin et al. (41) found no association of the *CYP1B1 L432V* allele with ovarian cancer risk in a study of 223 Caucasian cases and 280 controls. The present study, which is larger than either of these reports, found no association with ovarian cancer risk for the *L432V, R48G,* or *A199S* polymorphisms. No interactions were detected with smoking status or oral contraceptive use, and the observed interaction with parity was opposite to that reported by Goodman et al. (38). In this study, the *N453S* was genotyped but not analyzed, because the genotypes were not in HWE among the Caucasian control subjects. This absence of HWE remained even after

Table 5. Model-based groupings of estrogen bioactivation, SNP genotype combinations, and combined estimates of ovarian cancer risk among Caucasian subjects

Risk group	SNP genotype combinations in risk group*	No. subjects	OR† (95% CI)
Low	0-0-1-2, 0-0-2-1, 0-0-2-2, 0-1-0-0, 0-1-0-1, 0-1-1-0, 1-0-2-0, 1-1-1-0, 1-1-1-1, 1-1-1-2, 1-1-2-0, 1-1-2-1, 1-2-1-1, 2-0-1-0, 2-0-2-0, 2-1-1-1, 2-1-2-0, 2-1-2-1	205	0.71 (0.49-1.04)
Intermediate low	0-0-0-2, 0-0-1-1, 0-1-1-1, 1-0-0-2, 2-0-0-0, 2-0-0-1, 2-0-0-2	169	0.83 (0.55-1.23)
Neutral	0-0-2-0, 1-0-1-0, 1-0-1-1, 1-0-2-1, 1-1-0-2	244	1.0 (reference)
Intermediate high	1-0-0-0, 1-0-0-1, 1-0-1-2, 1-0-2-2, 2-0-1-1	214	1.44 (0.99-2.08)
High	0-0-0-0, 0-0-0-1, 0-0-1-0, 0-1-2-0, 0-1-2-1, 1-0-2-2, 1-1-0-0, 1-1-0-1, 2-0-1-2, 2-0-2-1, 2-0-2-2, 2-1-0-0, 2-1-0-1, 2-1-1-0, 2-2-1-0	162	1.57 (1.06-2.35)

*Number of copies of variant alleles of COMT-CYP1A1-CYP1B1-SULT1A1 SNPs.
†OR comparing individuals whose genotype combination is in the risk group of interest relative to those whose genotype combination is in the ''neutral'' risk group.

regenotyping using a different platform technology. Given the tight linkage disequilibrium between this SNP and the others included in the analysis, we should have been able to detect an association if one existed.

Three studies have been reported for the *COMT V158M* polymorphism and ovarian cancer. Goodman et al. (42) found no significant association in a case-control study (108 cases and 106 controls). This lack of association did not vary by age, family history, ovarian cancer histology, or *GSTT1* or *GSTM1* genotype. A study of Hawaiian subjects (38) also detected no main effect of this variant, although women who smoked and who had any *COMT Met* alleles had a borderline significant elevated risk of ovarian cancer (OR, 2.2; 95% CI, 1.0-4.7). This same report explored the interaction between *COMT* and *CYP1B1* and observed a 2.8-fold elevated risk for women who carried both a *CYP1B1 432Val* allele and a *COMT Met* group, although this interaction was not significant. Garner et al. (43) observed no main effect in their case-control study in New England of 240 ovarian cancer cases and 240 population-based controls, but carriers of the low-activity variant were at decreased risk of tumors with a mucinous histology (relative risk, 0.28; 95% CI, 0.13-0.61).

Although our data do not support the presence of strong associations between any individual SNP we studied and ovarian cancer risk, they do provide some evidence for the presence of an oligogenic association based on statistical measures of goodness-of-fit. However, when we constructed all 16 possible combinations of genotypes and estimated the corresponding ORs, we did not observe any semblance of monotonic increase in risk with increasing number of variant alleles. Because the sample size was relatively small compared with the frequencies of the genotype combinations, some of the multivariate-adjusted point estimates had very wide 95% CIs. We therefore attempted to simplify the oligogenic model by pooling genotype combinations into subgroups according to the risk of ovarian cancer as predicted by the oligogenic model. This exploratory analysis resulted in the formation of five subgroups of approximately equal size that retained the gradation in risk suggested by the full model while continuing to fit the observed data adequately. The identified groupings of SNP-associated ovarian cancer risk resulted in more stable estimates of disease risk. Although we do not expect all of the combined genotypes to share the same genetic risk or even that the identified classification is the optimal one, the outlined groupings provide some framework for summarizing which genotypic combinations seem to be associated with differential ovarian cancer risk. In addition to providing a relatively simple picture of classes of genotype combinations that are associated with differential risk for ovarian cancer, we expect that these groupings will provide a framework for future validation efforts.

Strengths of the study include the population-based ascertainment in the North Carolina site and the rapid case ascertainment used at both sites. The sample size is relatively large, permitting the construction of genotypes for the unit of analysis rather than comparison of allele frequencies between cases and controls. We had nongenetic risk factor data collected at both sites in a similar manner and were able to consider their influence in the statistical analysis. Our study sample was biracial in nature, affording some of the first data on the association of candidate susceptibility genes among African American subjects. The study participation rates were high, particularly among the cases, thereby contributing to study validity. The candidate genes are biologically plausible, and their selection was guided by data on the functional effects of the amino acid substitution encoded by the SNP. This not only enhanced interpretation of the study findings but also permitted the construction of an oligogenic model in which the high-risk alleles could be specified *a priori*.

Although this is the largest candidate gene study of ovarian cancer to date, there were still too few African American subjects to permit reliable estimates of risk. In addition, even among the Caucasian subjects, the estimates of risk in the oligogenic model become unstable. It is possible that the observed multigenic association with ovarian cancer risk was a chance finding. We examined several different single-SNP and multigenic associations, increasing the possibility of witnessing at least one type 1 error. Replication of these results in future studies will be needed to validate our observed findings. Recruitment at Mayo was not population based, but only cases in the immediate catchment area were included. Few data were available on potential differences between cases and controls on socioeconomic status. Although there was evidence that the cases had lower education levels than the controls, this is unlikely to reflect underlying genetic differences in the candidate genes investigated, because it has been shown that hospital-based case-control studies of candidate gene variation are equivalent to population-based studies (44). For each of the genes, we considered only SNPs in the coding exons. There is increasing interest in polymorphisms in regulatory regions of genes (45, 46), which will not affect enzyme activity but will affect the half-life of the encoded protein. Recently, it has been reported that promoter SNPs in *COMT* (47) and *SULT1A1* (48) that are in linkage disequilibrium with their open reading frame SNPs might better define their associations with risk of disease. Our present study did not include the promoter SNPs for those two genes. We are in the process of genotyping the study samples for those SNPs after which the intragene haplotype will be analyzed for associations with risk of ovarian cancer.

In summary, the current study provides modest evidence that polymorphisms in genes involved in catechol estrogen biosynthesis and degradation influence risk of epithelial ovarian cancer. Future studies should consider SNPs in both coding and regulatory regions to more fully evaluate and understand the inherited susceptibility hypotheses.

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