Risk of Testicular Germ Cell Cancer in Relation to Variation in Maternal and Offspring Cytochrome *P*450 Genes Involved in Catechol Estrogen Metabolism

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Abstract

The incidence of testicular germ cell carcinoma (TGCC) is highest among men ages 20 to 44 years. Exposure to relatively high circulating maternal estrogen levels during pregnancy has long been suspected as being a risk factor for TGCC. Catechol (hydroxylated) estrogens have carcinogenic potential, thought to arise from reactive catechol intermediates with enhanced capability of forming mutation-inducing DNA adducts. Polymorphisms in maternal or offspring genes encoding estrogen-metabolizing enzymes may influence prenatal catechol estrogen levels and could therefore be biomarkers of TGCC risk. We conducted a population-based, case-parent triad study to evaluate TGCC risk in relation to maternal and/or offspring polymorphisms in *CYP1A2*, *CYP1B1*, *CYP3A4*, and *CYP3A5*. We identified 18- to 44-year-old men diagnosed with invasive TGCC from 1999 to 2004 through a population-based cancer registry in Washington State and recruited cases and their parents (110 case-parent triads, 50 case-parent dyads). Maternal or offspring carriage of *CYP1A2* −163A was associated with reduced risk of TGCC [maternal heterozygote relative risk (RR), 0.6; 95% confidence interval (95% CI), 0.2-1.7; offspring heterozygote RR, 0.7; 95% CI, 0.3-1.5)]. Maternal *CYP1B1* ^{48}Gly homozygosity was associated with a 2.7-fold increased risk of TGCC (95% CI, 0.9-7.9), with little evidence that Leu^{432}Val or Asn^{453}Ser genotypes were related to risk. Men were also at increased risk of TGCC if they carried the *CYP3A4* −392G (RR, 7.0; 95% CI, 1.6-31) or *CYP3A5* 6986G (RR, 2.4; 95% CI, 1.1-5.6) alleles. These results support the hypothesis that maternal and/or offspring catechol estrogen activity may influence sons' risk of TGCC. (Cancer Epidemiol Biomarkers Prev 2005;14(9): 2183–90)

Introduction

Testicular germ cell carcinoma (TGCC) is the most common malignancy among younger men, with incidence peaking between ages 20 and 44 years (1). Apart from younger adulthood and being of Caucasian descent, a history of undescended testes (UDT) has been the only consistently observed risk factor for TGCC (1). The association with UDT, which has been associated with high gestational estrogen levels (2, 3), and the early age at onset of these tumors strongly suggest that tumor-initiating events occur during gestation, possibly by rendering germ cells susceptible to malignant transformation. Exposure to a relatively high circulating estrogen level during gestation has long been suspected as being a risk factor for TGCC (1, 2, 4, 5). Although data from animal models have strongly and consistently supported this hypothesis, epidemiologic studies have collectively produced equivocal results, possibly due to a reliance on self-reported surrogates of estrogen levels, such as pregnancy characteristics (1).

One mechanism by which *in utero* exposure to estrogens may influence the development of TGCC is via the effects of catechol metabolites of 17β-estradiol (E2) and estrone: 4-OH E2, 4-OH estrone, 2-OH E2, and 2-OH estrone. Catechol estrogens, which are produced by cytochrome *P*450 (CYP)–catalyzed hydroxylation of estrogens, readily undergo redox cycling between semiquinone and quinone forms through which reactive oxygen species (ROS) are produced. Oxygen radicals can directly damage DNA and can generate reactive intermediates that form mutation-inducing DNA adducts (6-8).

Mammalian adult male germ cells and supporting gonadal tissues are highly susceptible to the toxic effects of ROS (9-12), and catechol estrogens seem to be involved in some of these toxic effects (13, 14). Catechol estrogens and/or oxidative damage resulting from maternal sources could also contribute to testicular carcinogenesis *in utero*. Diethylstilbestrol, a known transplacental testicular carcinogen in animals, seems to exert its effects in part through its catechol and/or reactive quinone metabolites (15).

Variation in maternal or offspring genes encoding estrogen-metabolizing enzymes could influence the level of exposure to prenatal catechol estrogens and could therefore serve as biomarkers in a retrospective study of hormonal risk factors for TGCC. We conducted a population-based, case-parent triad study (16) to test whether TGCC risk is related to maternal and/or offspring variation in genes involved in catechol estrogen formation (*CYP1A2*, *CYP1B1*, *CYP3A4*, and *CYP3A5*). CYP1B1 and CYP3A5 are particularly active in the formation of 4-hydroxylated estrogens (17, 18). 4-OH catechol estrogen quinones form depurinating DNA adducts and seem to have greater transforming capability than 2-OH catechol estrogen quinones (8). We therefore hypothesized that maternal and/or offspring putative high-activity *CYP1B1* and *CYP3A5* variants would be associated with TGCC risk through increased 4-OH estrone and 4-OH E2 levels. In contrast, CYP1A2 and CYP3A4 tend to be more active in the 2-hydroxylation of estrogens (17, 19-21), and the putative

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high-activity *CYP1A2* and *CYP3A4* variants could be associated either with an increased risk of TGCC by increasing the overall pool of catechol estrogens or with a decreased risk of TGCC by increasing the amount of the less carcinogenic 2-OH estrogens relative to 4-OH estrogen levels.

Materials and Methods

As part of a population-based case-control study of risk factors for TGCC carried out in western Washington State, we conducted a case-parent triad study. Cases eligible for participation were all 18- to 44-year-old male residents of King, Pierce, and Snohomish counties, Washington State, diagnosed with invasive TGCC between January 1, 1999 and December 31, 2004, who had a residential telephone at diagnosis (because controls were ascertained through random-digit telephone dialing). We identified eligible TGCC cases from the files of the population-based Cancer Surveillance System of western Washington, one of the Surveillance, Epidemiology and End Results program registries.

As of January 1, 2005, we had approached 295 of 342 eligible TGCC cases. Forty-seven cases were not recruited as follows: they were unable to be located or were lost to follow-up ($n = 9$), had moved away ($n = 24$), had died ($n = 4$), or had physicians who did not permit us to contact them ($n = 10$). We offered \$50 to each case to participate in this study and recruited 262 cases (89% of approached cases and 77% of eligible cases).

We interviewed each consenting case in person regarding his demographic characteristics, medical history, family history, ethnic heritage, and lifestyle habits by using a structured questionnaire. Questions referred to events and behaviors before the month and year the case had been diagnosed with TGCC. From the files of the Cancer Surveillance System, we obtained data regarding each case's tumor's histologic type.

We asked each interviewed TGCC case to provide a blood specimen in EDTA-containing vacutainers and processed the specimen into buffy coats. If a TGCC case preferred not to have his blood drawn, we asked him to provide a sample of cells from the oral cavity by using a mouthwash technique or, in a few instances, by using four soft-bristle cytobrushes. We immediately placed buccal cell specimens on ice. We processed mouthwash samples to yield cell pellets (or cut off ends of cytobrushes) and froze them at −80°C. We obtained DNA from specimens by using salt precipitation and phenol/chloroform techniques (for buffy coats and mouthwash samples) or by using a QiaAmp mini kit (Qiagen, Inc., Valencia, CA) extraction system (for cytobrushes).

A TGCC case was eligible for inclusion in the case-parent triad study if he (*a*) provided a DNA sample, (*b*) reported not being adopted or reported being adopted but in contact with his genetic mother and/or father, and (*c*) had at least one living genetic parent (247 cases). A genetic mother or father of an eligible TGCC case was eligible if she or he was capable of communicating in English. As of January 1, 2005, we had been given permission to contact 78% of eligible parents of cases. We contacted each parent and offered him or her \$20 to participate in the study.

We interviewed consenting participating parents by telephone by using a structured, pretested questionnaire. We asked each parent about his or her race and ethnicity and asked mothers about pregnancy, medical, and lifestyle characteristics that may modify the relationship between TGCC risk and variation in *CYP1A2*, *CYP1B1*, *CYP3A4*, and/or *CYP3A5*. These factors included cigarette smoking, alcohol use, medication use, and exogenous estrogen exposure during the months when she was pregnant with the TGCC case. We collected biological specimens from parents in a similar fashion to those obtained from their sons, using express mail shipments for parents who lived outside the Seattle-Tacoma-Everett metropolitan area (~50% of the parents).

As of January 1, 2005, 92% of the 338 parents whom we had approached had completed interviews, and 4 of the interviewed parents refused to contribute a blood or buccal specimen. We have successfully obtained specimens (259 blood and 19 buccal) from 82% of the parents whom we have approached. Of these specimens, we have extracted DNA from and attempted genotyping of 272. The current results pertain to 160 cases and 270 parents (comprising 110 triads and 50 dyads) whose specimens underwent DNA extraction and successful genotyping for at least one of the six polymorphisms. Of the dyads, approximately two-thirds are mother-son pairs and the remaining third father-son pairs.

Molecular Genotyping Methods. For each locus, we genotyped participants for single nucleotide polymorphisms (SNP) for which there was evidence of a correlation with functional activity or estrogen-related outcomes at the time we initiated the study: three SNPs in *CYP1B1* (Ala^{48}Gly caused by 142C>G, rs10012; Leu^{432}Val caused by 4326C>G, rs1056836; and Asn^{453}Ser caused by 4390A>G, rs1800440) and one SNP each in *CYP1A2* (−163C>A, rs762551), *CYP3A4* (−392A>G, rs2740574), and *CYP3A5* (6986A>G, rs776746; http://www.imm.ki.se/CYPalleles). All assays were conducted blinded as to status (case, mother, or father) or any other characteristics of the participant.

We used ABI Prism SNaPshot multiplex assays to determine genotypes. We first amplified a gene-specific PCR product containing the SNP by using PCR primers and cycling conditions for each gene.[6] After amplification, the PCR products were pooled in a 1:1 ratio and treated with 5 units shrimp alkaline phosphatase (U.S. Biochemical Corp., Cleveland, OH) and 2 units exonuclease I (New England Biolabs, Beverly, MA) at 37°C for 1 hour followed by heat inactivation at 75°C for 15 minutes. We then used the pooled, treated PCR products as a template for the SNaPshot reaction. We simultaneously analyzed three SNPs in each injection by using probes complementary to the specific templates with the addition of a nonhomologous tail (GATC), of different length for each polymorphism, to the sequence immediately 5′ of the SNP site. The SNaPshot reaction contained 3 μL pooled PCR products, 5 μL of 2× SNaPshot Multiplex Ready Reaction Mix, and 0.02 to 0.1 μmol/L each probe. Cycling of the SNaPshot reaction was as follows: 25 cycles of 96°C for 10 seconds, 50°C for 5 seconds, and 60°C for 30 seconds. The probes annealed to their respective PCR products, and the SNaPshot reaction extended each annealed probe by a single fluorescently labeled dideoxyribonucleoside triphosphate over the site of the SNP. Each of the four dideoxyribonucleoside triphosphates was labeled with a different fluorescent dye. Subsequently, this reaction was treated with 1 unit shrimp alkaline phosphatase for 1 hour at 37°C followed by heat inactivation at 75°C for 15 minutes. This multiplex reaction (0.5 μL) was combined with 0.1 μL LIZ-120 size standard (Applied Biosystems, Foster City, CA) and 9.4 μL formamide, heat denatured, and run on an ABI 3100 Genetic Analyzer. We assigned genotypes by using GeneMapper (Applied Biosystems) software, and two experienced staff confirmed the results by independent inspection. In case of disagreements, we repeated the assays or tested the samples with an alternative method until we obtained unequivocal results. Positive and negative controls were included in each run. Blinded multiple replicate DNA preparations were included on all plates (42 or 43 replicate sets for each of the *CYP1A2*, *CYP1B1*, and *CYP3A5* SNPs and 95 replicate sets for the *CYP3A4* SNP, resulting in 308 person-genotypes that could

[6] Supplementary data are available at http://cebp.aacrjournals.org.

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be compared); one sample yielded discrepant results for one allele at the *CYP3A5* SNP and an error rate of 0.3% (this discrepancy pertained to a case not included in the family study).

Data Analysis. We identified two case-parent triads exhibiting inconsistency with Mendelian inheritance at the *CYP1B1* Ala[48]Gly locus. In neither of these triads was there evidence of inconsistency at 14 other loci for which genotype data were available (only 5 of which are included in this report). Although the chances are very low that truly unrelated individuals would share genotypes at the 14 other genetic loci, we excluded these two families from analyses of the *CYP1B1* Ala[48]Gly locus. One additional triad exhibited inconsistency with Mendelian inheritance at both the *CYP1B1* Asn[453]Ser locus and another locus not included in this report; we excluded this triad from all analyses. Although such exclusions can bias the results of the analysis, the exclusion of only one triad on this basis should not meaningfully affect our results (22).

We used a log-linear case-parent triad approach to assess the association between genetic polymorphisms and TGCC risk by estimating separately, in a log-linear regression model, the relative risk [RR; 95% confidence intervals (95% CI)] associated with the mother's or son's having one or two copies of the allele of interest at a given locus (16, 23). The log-linear approach conditions on the combination of parental genotypes (or ''mating types'') and thereby renders independent tests of disease associations with maternal and offspring genotypes. This conditioning also makes it unnecessary to assume that alleles are in Hardy-Weinberg equilibrium. Maternal genotype RRs can be estimated similarly to a matched-pair design in which each case's mother is matched to his father. The maternal RR is estimated among sets in which mothers and fathers have discordant genotypes, as a departure from the expected proportion (50%) of mothers and fathers each having the genotypes with the higher number of ''variant'' alleles. Offspring genotype RRs can be estimated as a departure from the expected proportion (50%) inheriting a given allele from heterozygous parents. For estimation of offspring RRs, this approach is a generalization of the transmission disequilibrium test. We tested statistically whether the mother's or offspring's genotype at each locus was associated with TGCC by performing a likelihood ratio test comparing the model estimating maternal (or offspring) associations and that in which maternal (or offspring) associations with TGCC risk were not modeled. We do not present the results in terms of statistical significance and therefore did not adjust the resulting *P*s to account for the multiple statistical tests done. The likelihood ratio test had 1 *df* (for *CYP3A4* or *CYP3A5* offspring heterozygotes RRs) or 2 *df* (all other association estimates). When genetic information was available from only one parent, we estimated associations by applying an expectation-maximization algorithm approach to the regression modeling (24, 25).

To account for the possibility that genetic risk factors for TGCC vary by histologic type, we did analyses separately for cases diagnosed with seminomas and those with tumors characterized histologically as nonseminomas. Genetic associations with TGCC risk may also be modified by ethnic background, by the case's having a medical history of UDT, or by family history of TGCC. In addition, log-linear case-parent triad–based estimates of maternal RRs (but not offspring RRs) may be sensitive to confounding by ethnicity. For these reasons, we repeated the analyses among (*a*) sets in which no member was of non-Caucasian race or Hispanic ethnicity, (*b*) sets in which the case had no history of UDT, and (*c*) sets in which the case reported no family history of TGCC. Because RRs associated with genetic factors are sometimes higher among cases with relatively early onset, we did analyses separately among sets in which the age of onset was <34 years (the median age of onset) and those in which the age of onset was ≥34 years.

Associations between CYP variants and TGCC risk may depend on factors that influence the activity of these enzymes or, more generally, estrogen bioavailability or activity. There were too few exposed sets, however, to evaluate formally how associations were modified by maternal or sons' characteristics. Thus, we repeated analyses restricted to sets in which mothers had not reported any exposure to specific factors or specific conditions during the pregnancy with the case. These factors included use of exogenous estrogens, smoking more than three cigarettes per day, drinking more than two drinks per week, gestational diabetes, or medications known to induce or inhibit CYP1A2, CYP1B1, and/or CYP3A4 (26-28). We also repeated analyses excluding sets in which the case had reported that before his diagnosis with TGCC he had a cigarette smoking history of ≥10 pack-years cumulatively or had taken medications known to influence CYP1A2 and/or CYP3A4 activities for at least 3, 6, or 12 months cumulatively. We did these subanalyses in three ways: (*a*) by excluding sets in which only the son (before TGCC diagnosis) or only the mother (during the pregnancy with the case) was exposed to one potential effect modifier, separately for each factor and family member's exposure; (*b*) by excluding sets in which the case's mother was exposed to any potential effect modifier during her pregnancy with the case; and (*c*) by excluding sets in which the case (before diagnosis), his mother (during her pregnancy), or both was exposed to any potential effect modifier.

We did all regression analyses in the software program LEM (25).

Results

Data were available on 160 case-parent sets, of which 110 were complete triads. Of the participating families, two-thirds involved cases diagnosed with seminomas, with the majority diagnosed when the malignancy was still localized to the testes (Table 1). The average age of cases at the time of their TGCC diagnosis was 33 years (Table 1). Twelve (7.5%) cases reported a history of UDT (Table 1), and 6 (3.8%) reported that a brother, father, or grandfather had been diagnosed with TGCC. These characteristics generally did not differ, on average, among cases participating in this case-parent study compared with all eligible TGCC cases diagnosed during the eligibility period. The proportion with seminomas was slightly higher (64% versus 61%) among the cases described in this report.

Table 1. Selected medical characteristics of TGCC patients, by tumor histology

Characteristics	All histologic types (*n* = 160) *n* (%)	Seminoma (*n* = 103) *n* (%)	Nonseminoma (*n* = 57) *n* (%)
Stage at diagnosis			
Localized	121 (75.6)	88 (85.4)	33 (57.9)
Regional	27 (16.9)	12 (11.7)	15 (26.3)
Distant	12 (7.5)	3 (2.9)	9 (15.8)
Age at diagnosis (y)			
18-24	25 (15.6)	10 (9.7)	15 (26.3)
25-29	30 (18.8)	14 (13.6)	16 (28.1)
30-34	33 (20.6)	22 (21.4)	11 (19.3)
35-39	32 (20.0)	26 (25.2)	6 (10.5)
40-44	40 (25.0)	31 (30.1)	9 (15.8)
Mean (SD)	33.0 (7.0)	35.3 (6.0)	30.2 (7.5)
History of UDT			
Yes	12 (7.5)	8 (7.8)	4 (7.0)
No	147 (91.9)	94 (91.3)	53 (93.0)
Do not know	1 (0.6)	1 (1.0)	0 (0)

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Mothers and fathers were, on average, ages 26 and 28 years, respectively, when the participating son was born (range, 17-41 years for mothers and 19-46 years for fathers). The average ages of mothers and fathers at the time of the telephone interview were 61 and 63 years, respectively (range, 43-82 years for mothers and 44-83 years for fathers).

All participants reported being Caucasian, of whom >95% of cases, mothers, or fathers reported being solely of Caucasian descent and non-Hispanic ethnicity. Twenty-two case-parent sets included at least one family member who additionally reported being of non-White race (usually American Indian) or Hispanic ethnicity.

Mating types 2, 3, and 5 (Table 2), in which the parents have discordant genotypes, are informative regarding maternal RRs. Mating types 2, 4, and 5, in which either (or both) parent is heterozygous (Table 2), are informative regarding offspring RRs. The number of informative case-parent sets varied from 17 at the *CYP3A4* locus to 76 for *CYP1B1* Leu^{432}Val offspring RRs (Table 2). This number was usually similar for the maternal and offspring RRs at a given locus.

Maternal or offspring carriage of the *CYP1A2* −163A allele was associated with a decreased risk of TGCC (maternal heterozygote RR, 0.6; 95% CI, 0.2-1.7; homozygote RR, 0.9; 95% CI, 0.3-2.3; offspring heterozygote RR, 0.7; 95% CI, 0.3-1.5; homozygote RR, 0.5; 95% CI, 0.2-1.2; Table 3).

Maternal carriage of the *CYP1B1* ^{48}Gly allele was associated with an increased risk of TGCC (heterozygote RR, 1.3; 95% CI, 0.7-2.2; homozygote RR, 2.7; 95% CI, 0.9-7.9), whereas offspring carriage of the same allele was unassociated with the risk of TGCC (Table 3).

At the *CYP3A* loci, RRs for maternal carriage of postulated high-risk variants tended to be <1 or close to 1, whereas offspring who were heterozygous for either *CYP3A4* −392G or *CYP3A5* 6986A allele were at an increased risk of TGCC (*CYP3A4* RR, 7.0; 95% CI, 1.6-31; *CYP3A5* RR, 2.4; 95% CI, 1.1-5.6). The low variant allele frequency rendered both maternal and offspring estimates imprecise, although inclusion of dyads greatly reduced the width of the offspring 95% CI as well as slightly attenuating the RRs (Table 3).

The RR estimates differed only very slightly among sets involving cases with seminoma and those involving cases with nonseminomas (data not shown). In analyses excluding 22 families (of which 14 were triads) because at least one member reported being non-Caucasian and/or of Hispanic ethnicity, the RR estimates were at most only slightly altered (data not shown). Neither maternal nor offspring RR estimates were affected materially by excluding from the analyses either (*a*) the six sets in which the case had reported a male relative's having been diagnosed with TGCC or (*b*) the 12 sets (eight triads) in which the case reported a positive or unknown history of UDT. The RR estimates also did not differ meaningfully regarding cases with earlier or later ages at diagnosis of TGCC.

Excluding case-parent sets in which either the case or the case's mother reported exposures to known inducers or inhibitors of *CYP1A2*, *CYP1B1*, and/or *CYP3A4* expression generally affected the magnitude of the RRs and *P*s only slightly. The greatest differences compared with the primary analyses involved the *CYP1B1* offspring Leu^{432}Val genotypes: offspring heterozygosity and homozygosity for the ^{432}Val allele were 1.9 (95% CI, 0.7-4.4) and 3.3 (95% CI, 1.2-8.9), respectively (P = 0.17) among triads only, after excluding 28 triads in which either the case or his mother reported exposures to CYP1B1-influencing medications, positive cigarette smoking history, or (for the mother) pregnancy exposure to exogenous estrogens (data not shown).

Table 2. Observed and expected triad genotype distributions among TGCC case-parent triads, by mating type and locus

Mating type*	Gene and polymorphism											
M,F,C†	*CYP1A2* −163C>A		*CYP1B1* Arg^{48}Gly		*CYP1B1* Leu^{432}Val		*CYP1B1* Asn^{453}Ser		*CYP3A4* −392A>G		*CYP3A5* 6986A>G	
	Observed	Expected	Observed	Expected	Observed	Expected	Observed	Expected	Observed	Expected	Observed	Expected
1												
2,2,2	25	25	0	0	5	5	0	0	0	0	0	0
2												
2,1,2	10	10.25	2	2	4	6	0	0.25	0	0.25	0	0.25
2,1,1	14	10.25	3	2	8	6	1	0.25	1	0.25	1	0.25
1,2,2	7	10.25	1	2	6	6	0	0.25	0	0.25	0	0.25
1,2,1	10	10.25	2	2	6	6	0	0.25	0	0.25	0	0.25
3												
2,0,1	6	6	7	4.5	9	7.5	1	2.5	0	0.5	0	1
0,2,1	6	6	2	4.5	6	7.5	4	2.5	1	0.5	2	1
4												
1,1,2	3	4.5	4	3.25	13	8	0	1.75	0	0	0	0.25
1,1,1	10	9	4	6.5	9	16	4	3.5	0	0	1	0.5
1,1,0	5	4.5	5	3.25	10	8	3	1.75	0	0	0	0.25
5												
1,0,1	1	1.5	13	11	4	5	4	8.75	7	4	8	6.75
1,0,0	1	1.5	11	11	2	5	12	8.75	2	4	5	6.75
0,1,1	0	1.5	9	11	8	5	10	8.75	7	4	11	6.75
0,1,0	4	1.5	11	11	6	5	9	8.75	0	4	3	6.75
6												
0,0,0	1	1	27	27	8	8	56	56	89	89	70	70
Total	103	103	101	101	104	104	104	104	107	107	101	101
Maternal, RR-informative	59		61		59		41		18		30	
Offspring, RR-informative	65		65		76		43		17		29	
Definition of genotypes for each locus												
0	CC		CC		CC		AA		AA		GG	
1	CA		CG		CG		AG		AG		GA	
2	AA		GG		GG		GG		GG		AA	

NOTE: Expected numbers of triads under the null hypothesis that neither maternal nor offspring genotypes are associated with risk of TGCC.
*Mating types 2, 3, and 5 are informative for maternal RRs; mating types 2, 4, and 5 are informative for offspring RRs.
†M, F, and C denote maternal, paternal, and case genotypes, respectively.

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Table 3. RR of TGCC associated with maternal and offspring genotypes among case-parent triads and dyads, by locus

	Gene and polymorphism					
	CYP1A2 −163C>A	*CYP1B1* Arg^{48}Gly	*CYP1B1* Leu^{432}Val	*CYP1B1* Asn^{453}Ser	*CYP3A4* −392A>G	*CYP3A5* 6986A>G
Triads only						
Maternal (*n*)	103	101	104	104	107	101
No. informative triads	59	61	59	41	18	30
Reference	1.0	1.0	1.0	1.0	1.0	1.0
Heterozygote RR	0.6 (0.2-1.7)	1.3 (0.7-2.2)	0.7 (0.3-1.4)	0.8 (0.4-1.5)	1.1 (0.4-3.0)	0.8 (0.4-1.8)
Homozygote RR	0.9 (0.3-2.3)	2.7 (0.9-7.9)	0.9 (0.4-2.0)	0.5 (0.1-2.6)	1.1 (0.1-18)	0.5 (0.0-5.3)
*P**	0.045	0.086	0.449	1.000	1.000	1.000
Offspring						
No. informative triads	65	65	76	43	17	29
Reference	1.0	1.0	1.0	1.0	1.0	1.0
Heterozygote RR	0.7 (0.3-1.5)	0.9 (0.5-1.5)	1.0 (0.5-1.7)	0.7 (0.4-1.2)	7† (1.6-31)	2.4† (1.1-5.6)
Homozygote RR	0.5 (0.2-1.2)	0.9 (0.3-2.4)	1.2 (0.6-2.6)	Undefined	Undefined†	Undefined†
*P**	0.135	0.849	1.000	0.077	0.011	0.110
Triads and dyads						
Maternal (*n*)	153	150	153	153	158	151
Reference	1.0	1.0	1.0	1.0	1.0	1.0
Heterozygote RR	0.4 (0.2-1.0)	1.4 (0.8-2.3)	0.6 (0.3-1.1)	0.9 (0.5-1.7)	0.9 (0.4-2.1)	0.8 (0.4-1.5)
Homozygote RR	0.6 (0.3-1.5)	2.6 (1.1-6.3)	0.8 (0.4-1.5)	0.4 (0.1-2.1)	0.9 (0.1-14)	0.4 (0.0-4.2)
*P**	0.010	0.005	0.156	0.897	0.962	1.000
Offspring						
Reference	1.0	1.0	1.0	1.0	1.0	1.0
Heterozygote RR	0.7 (0.3-1.4)	0.9 (0.5-1.4)	1.2 (0.7-2.0)	0.8 (0.5-1.3)	5.2† (1.5-18)	1.8† (0.9-3.6)
Homozygote RR	0.5 (0.2-1.2)	1.0 (0.4-2.4)	1.3 (0.6-2.6)	0.2 (0.0-1.4)	Undefined†	Undefined†
*P**	0.079	0.677	1.000	0.273	0.013	0.256
Definition of genotypes						
Reference group	CC	CC	CC	AA	AA	GG
Heterozygote	CA	CG	CG	AG	AG	GA
Homozygote	AA	GG	GG	GG	GG	AA

*To calculate *P*s, we calculated twice the difference between the log likelihood of the model estimating maternal effects and that in which maternal (or offspring) associations with offspring TGCC risk are assumed to be zero and compared the result to a χ^2 distribution with 1 *df* (*CYP3A4* or *CYP3A5* offspring heterozygote RRs) or 2 *df* (all other loci).

† Because we observed no offspring who were homozygous for either the *CYP3A4* −392G allele or the *CYP3A5* 6986A allele, we estimated the offspring associations imposing a dominant genetic model.

Discussion

Hormones may have a bearing on the incidence of TGCC, the illness possibly developing in the setting of high prenatal estrogen exposure (1). Because estrogen and its metabolites cross the placenta freely, maternally mediated catechol estrogen metabolism could affect the level of fetal exposures throughout pregnancy. Studies in human tissue *in vivo* and *in vitro* suggest that maternal and fetal blood supplies contain similar levels of catechol estrogens (29). Fetal deactivation of these compounds through methylation is reduced compared with maternal deactivation (29), however, highlighting the potential importance of maternal enzyme activity in regulating fetal exposures. That fetal susceptibility to the effects of ROS can be modified by maternal physiology has been shown indirectly in two different animal models of pregnancy: (*a*) in that pregnant females lacking activity of enzymes involved in ROS detoxification (e.g., glucuronidase and glutathione peroxidase) have an increased risk of developing damaged fetuses and (*b*) in that administration of antioxidant or free radical-quenching compounds to pregnant animals reduces the teratogenicity of chemicals known to produce ROS-related DNA damage (30). Furthermore, in a recent epidemiologic study, mothers of TGCC cases had higher circulating levels of CYP enzyme-inducing polychlorinated biphenyl compounds than mothers of age-matched controls (31), providing indirect support for the idea that maternally mediated enzymes involved in ROS production influence testicular carcinogenesis. In contrast, levels of the same polychlorinated biphenyl compounds in blood samples from cases and controls were unassociated with TGCC risk. Variation in both maternal and sons' own genes could be expected to influence exposure to estrogen metabolites during sons' gestation or postnatally.

In this population-based case-parent triad study, maternal and offspring carriage of the *CYP1A2* −163A allele were associated with an ~10% to 50% reduction in the risk of TGCC. The *CYP1A2* −163A allele has more often been associated with increased enzymatic activity, which would lead to higher catechol estrogen formation (32-34). The association we observed is consistent with a recently conducted case-control study of TGCC (35). In that study, men in the highest tertile of CYP1A2 activity, measured as a ratio of urinary metabolites of dietary caffeine, were at one-third the risk of TGCC compared with men in the lowest tertile. Furthermore, there are at least three mechanisms through which higher CYP1A2 activity could decrease the risk of TGCC.

First, because of a strong preference for 2-hydroxylation, increased CYP1A2 activity may decrease TGCC risk by shifting estrogen metabolism toward an increased ratio of 2:4 hydroxylated products. It is thought that 2-hydroxylated estrogens have much less mutagenic potential than the 4-hydroxylated forms, both because of the instability of the DNA adducts formed by 2-hydroxylated estrogens (36) and because 2-hydroxylated estrogens lack traditional hormonal action via the estrogen receptor (17). Indeed, among hepatically expressed CYP enzymes, CYP1A2 has the greatest activity for the 2-hydroxylation of estrogens (21, 37). The stability of 2-OH and 4-OH estrogens also depends on their relative rates of detoxification, however, suggesting that other enzymes could also affect the ratio of circulating 2:4-OH estrogens and catechol estrogen levels overall (38). Second, increased CYP1A2 activity may simply decrease the pool of bioavailable estrogens, which themselves exert growth-related activities that may mediate the transformation of germ cells (17, 38). Third, CYP1A2 activity may influence TGCC risk through a pathway that does not involve steroid hormones, as this

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enzyme is involved in the activation or detoxification of a wide variety of endogenous and exogenous compounds, including polyaromatic hydrocarbons, polychlorinated biphenyl compounds, and many medications (28). It could be that mothers with lower CYP1A2 activity expose gestating offspring to higher levels of transplacental carcinogens that might otherwise be detoxified.

Alternatively, the association we observed between maternal or offspring *CYP1A2* −163 genotypes and TGCC risk may be due either to chance or to linkage disequilibrium with other genetic variants. The −163A allele has been reported to be in linkage disequilibrium with a G−2964A variant in Chinese individuals (39). In two studies in Asian individuals genotyped at both loci, the −2964 GG genotype was associated with higher CYP1A2 activity (39, 40). Among haplotypes identified in Ethiopians and characterized by reporter gene constructs, the variant that was most strongly supported as a functionally relevant allele (C−730T) was in linkage disequilibrium with the −163 polymorphism, although it was also much rarer than the −163 variant (41). In addition, *CYP1A2* variants are in linkage disequilibrium with *CYP1A1* alleles, which themselves have been associated with the risk of hormonally related cancers (42-44). Moreover, CYP1A1 and CYP1A2 share many of the same enzymatic activities and may be under coordinated regulation (45), which complicates efforts to study them independently. Placental expression and activity of CYP1A1 seem to be greater than those of CYP1A2 and to occur earlier in the pregnancy (46), but we did not evaluate any *CYP1A1* variants in this study.

Of the three different *CYP1B1* SNPs, the most robust results regarding maternal genotypes are for the ^{48}Gly substitution, which was associated with a 1.3- and 2.7-fold increase in risk of TGCC in maternal heterozygotes and homozygotes, respectively, compared with ^{48}Ala homozygotes. Much less is known about this substitution than about the ^{432}Val and ^{453}Ser variants, but it has been reported to be unassociated with CYP1B1-mediated E2 hydroxylation *in vitro* activity (47, 48) and with breast cancer risk (49). We observed little overall association with maternal or offspring genotypes at Asn^{453}Ser or Leu^{432}Val. The latter is the best characterized *CYP1B1* variant; ^{432}Val has most often been correlated with higher activity (47, 50-52), an enhanced preference for the 4-hydroxylation of estrogen (51, 53), and an increased risk of estrogen-related health outcomes (50, 53-56) although not in all studies in which these associations were evaluated (44, 49, 53, 55, 57, 58). Among cases who had not been exposed to CYP1B1 inducers directly or transplacentally, however, the *CYP1B1* ^{432}Val allele was associated with a 2- to 3-fold increased risk of TGCC. These differences from the unstratified analyses may be likely to reflect sampling differences, as the resulting sample size included only 76 complete triads, of which 52 were informative. Furthermore, previously published evaluations of a similar gene-environment interaction have suggested that the RR associated with the ^{432}Val allele is lower (not higher) among those unexposed to particular inducers (55, 56).

CYP3A4 and CYP3A5 are structurally related enzymes that share most substrate activities. CYP3A5 is expressed at high levels in only ~15% of Caucasians. It is generally thought to have lower activity than CYP3A4 for most substrates, except that it seems to have greater regiospecificity for the 4-hydroxylation of estrone and E2 (18, 37). Maternal genotypes were only weakly associated with TGCC risk, but sons carrying either variant, all of whom were heterozygous, were at an increased risk of TGCC. The latter finding is consistent with evidence that the *CYP3A5* 6986A>G substitution leads to incorrect mRNA splicing and lack of functional protein (59, 60). CYP3A4 (in particular; ref. 61) and CYP3A5 also inactivate testosterone by 6β-hydroxylation, and increased CYP3A4 could therefore cause a decrease in bioavailable testosterone. Reduced androgen support for the developing testes and germ cells has also been hypothesized to be a risk factor for TGCC (62). In a recent study, pregnant African American mothers, whose sons are at substantially lower risk of TGCC compared with White men, were found to have higher blood testosterone than pregnant White women, whereas blood estrogen levels were similar (63). It is therefore difficult to tease out whether the association with *CYP3A* genotypes represents associations with catechol estrogen formation, testosterone metabolism, or both. Moreover, because the *CYP3A4* and *CYP3A5* variants we studied are in linkage disequilibrium ($r^2 \approx 0.5$ in our sample; ref. 60), larger studies will be needed to determine whether the disease associations we observed are mutually independent, particularly because recent data on the *CYP3A4* −392G>A polymorphism have raised doubts about the functional significance of the variant allele (26).

Because of the very low variant allele frequencies (5-10%), genotypes at these two *CYP3A* loci were most susceptible to the study's relatively small sample size. We could not estimate the RR associated with offspring homozygosity for either variant allele, and all RR estimates were imprecise due to the small number of informative triads: 17 and 29 at the *CYP3A4* and *CYP3A5* loci, respectively. Moreover, simulation studies have shown that the likelihood ratio test of genotype associations was probably conservative and the heterozygote RR estimates potentially biased toward the null (64). Among the other loci, the number of informative triads ranged from 41 to 76, allowing for more stable estimates that were likely to have been unbiased. Still, the expected statistical power to detect RRs ~2-fold ranged from <10% to ~50% depending on the true genetic model (dominant, recessive, etc.) and the high-risk allele frequency.

To increase power, we used an expectation-maximization algorithm method to incorporate information from sets in which we had genotyped only one parent. This was particularly useful for estimating offspring RRs, because some dyads included heterozygous parents and thus contributed additional information. At large sample sizes, genotype information from additional dyads theoretically should not alter maternal RR estimates but can improve the precision of these estimates. In our expectation-maximization–based analyses, however, most maternal RR estimates shifted slightly from the complete-triad analyses. This could indicate that some expectation-maximization–based results are unstable due to the small sample size.

Activities of these highly inducible CYP enzymes may fluctuate greatly in response to a wide variety of endogenous and exogenous exposures, including steroid hormones, dietary factors, many medications, and chemicals in cigarette smoke or air pollution. To evaluate possible effect modification, we limited these assessments to the largest subgroups: those with no reported exposure to CYP enzyme inducers or inhibitors. Such analyses produced RR estimates that were generally altered only very slightly. Because the number of excluded sets was fairly small, however, we would have expected large perturbations of the RRs only if there were very strong associations in the sets with reported exposure to specific compounds. Larger sample sizes will be needed for more formal and rigorous assessments of effect modification by nongenetic factors.

Finally, two implicit assumptions of the case-parent triad method we applied are that genotypes are (*a*) unrelated to prenatal or postnatal survival (which could bias the offspring RRs) and (*b*) unrelated to differences in survival or the participation rates of mothers compared with fathers (which could bias the maternal RRs). The former assumption implies the need to follow up any positive findings in population-based

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case-control studies. The latter assumption seems unlikely to have accounted for the associations we observed between TGCC risk and maternal genotypes (64).

The role of *in utero* estrogen exposure in TGCC etiology has been a target of epidemiologic inquiry for >25 years. We have examined offspring TGCC risk in relation to maternal genetic polymorphisms in catechol estrogen-metabolizing genes, perhaps the first investigation incorporating maternal genetic markers to study an aspect of the *in utero* estrogen hypothesis. The sample size may have limited our ability to draw strong conclusions regarding the broader catechol estrogen pathway. Nevertheless, these results support the hypothesis that levels of catechol estrogens, either in the maternal or in the son's own circulation, may influence the risk of TGCC in sons.

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Risk of Testicular Germ Cell Cancer in Relation to Variation in Maternal and Offspring Cytochrome *P*450 Genes Involved in Catechol Estrogen Metabolism

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