# Breast Cancer Risk Reduction and Membrane-Bound Catechol *O*-Methyltransferase Genetic Polymorphisms

Yuan Ji,[1] Janet Olson,[2] Jianping Zhang,[1] Michelle Hildebrandt,[1] Liewei Wang,[1] James Ingle,[3] Zachary Fredericksen,[2] Thomas Sellers,[4] William Miller,[5] J. Michael Dixon,[5] Hiltrud Brauch,[6] Michel Eichelbaum,[6] Christina Justenhoven,[6] Ute Hamann,[7] Yon Ko,[8] Thomas Brüning,[9] Jenny Chang-Claude,[7] Shan Wang-Gohrke,[10] Daniel Schaid,[2] and Richard Weinshilboum[1]

[1]Division of Clinical Pharmacology, Department of Molecular Pharmacology and Experimental Therapeutics, [2]Department of Health Sciences Research, [3]Department of Oncology, Mayo Clinic College of Medicine, Mayo Clinic, Rochester, Minnesota; [4]H. Lee Moffitt Cancer Center, Tampa, Florida; [5]Western General Hospital, Edinburgh, Scotland; [6]Dr. Margarete Fischer-Bosch-Institut für Klinische Pharmakologie, Stuttgart, and Tübingen Universität, Tübingen, Germany; [7]German Cancer Research Center, Heidelberg, Germany; [8]Johanniter Hospital Bonn, Bonn, Germany; [9]Berufsgenossenschaftliches Forschungsinstitut für Arbeitsmedizin, Ruhr Universität Bochum, Bochum, Germany; and [10]Women's Hospital of the University of Ulm, Ulm, Germany

## Abstract

Catechol *O*-methyltransferase (COMT)-catalyzed methylation of catecholestrogens has been proposed to play a protective role in estrogen-induced genotoxic carcinogenesis. We have taken a comprehensive approach to test the hypothesis that genetic variation in *COMT* might influence breast cancer risk. Fifteen *COMT* single nucleotide polymorphisms (SNPs) selected on the basis of in-depth resequencing of the *COMT* gene were genotyped in 1,482 DNA samples from a Mayo Clinic breast cancer case control study. Two common SNPs in the distal promoter for membrane-bound (MB) COMT, rs2020917 and rs737865, were associated with breast cancer risk reduction in premenopausal women in the Mayo Clinic study, with allele-specific odds ratios (OR) of 0.70 [95% confidence interval (CI), 0.52–0.95] and 0.68 (95% CI, 0.51–0.92), respectively. These two SNPs were then subjected to functional genomic analysis and were genotyped in an additional 3,683 DNA samples from two independent case control studies (GENICA and GESBC). Functional genomic experiments showed that these SNPs could up-regulate transcription and that they altered DNA-protein binding patterns. Furthermore, substrate kinetic and exon array analyses suggested a role for MB-COMT in catecholestrogen inactivation. The GENICA results were similar to the Mayo case control observations, with ORs of 0.85 (95% CI, 0.72–1.00) and 0.85 (95% CI, 0.72–1.01) for the two SNPs. No significant effect was observed in the GESBC study. These studies showed that two SNPs in the *COMT* distal promoter were associated with breast cancer risk reduction in two of three case control studies, compatible with the results of functional genomic experiments, suggesting a role for MB-COMT in breast cancer risk. [Cancer Res 2008;68(14):5997–6005]

## Introduction

Breast cancer is the most common cancer and the second leading cause of cancer death in women (1). Lifetime estrogen exposure is a major risk factor for breast cancer (2, 3). Estrogens can potentially induce carcinogenesis through both receptor-mediated and nonreceptor-mediated pathways (Fig. 1; refs. 4–6). This latter pathway is thought to involve metabolic activation, during which estrogens are metabolized by cytochrome P450 enzymes to form catecholestrogens, which can then be converted to estrogen quinones capable of forming stable or depurinating DNA adducts, resulting in genotoxicity (5, 7). Because 4-OH catecholestrogens are also potent estrogen receptor (ER) ligands (8, 9), catecholestrogens could potentially influence both pathways for estrogen carcinogenesis. Catechol *O*-methyltransferase (EC2.1.1.6; COMT) catalyzes the methylation of catechols, including catecholestrogens (10, 11). In addition, 2-methoxy catecholestrogens also have strong anticarcinogenic effects (12). Therefore, decreased COMT activity might be a risk factor for breast cancer, whereas elevated activity would, in theory, be protective.

*COMT* encodes two isoforms, a soluble cytoplasmic (S-COMT) and a membrane-bound isoform (MB-COMT; refs. 13, 14; Fig. 2*A*). A "proximal promoter" in intron 2 drives S-COMT transcription, whereas a "distal promoter," 20 kb upstream at the 5′ end of the gene, does the same for MB-COMT (13, 14). The two COMT isoforms differ only by an additional 50 hydrophobic amino acids at the $NH_2$ terminus of MB-COMT (13, 14). MB-COMT is believed to be the dominant isoform in the brain, whereas S-COMT predominates in peripheral tissues (13–15).

We first described the autosomal codominant inheritance of S-COMT activity 30 years ago (16, 17), and its genetic basis was subsequently shown to result, in part, from a nonsynonymous G to A polymorphism that changes a Val to Met at codon 108 for S-COMT and codon 158 for MB-COMT (18). The Met108/158 allele is associated with decreased enzyme activity and decreased thermal stability, both *in vitro* and *in vivo* (16, 17, 19–21). During the past decade, >40 epidemiologic studies have been performed to test the possible association of *COMT* sequence variation with breast cancer risk. Nearly all of those studies have focused on only the Val108/158Met polymorphism, but the results have been inconsistent (22–24).

In the present study, we took a systematic approach to test the hypothesis that genetic polymorphisms in *COMT* might influence
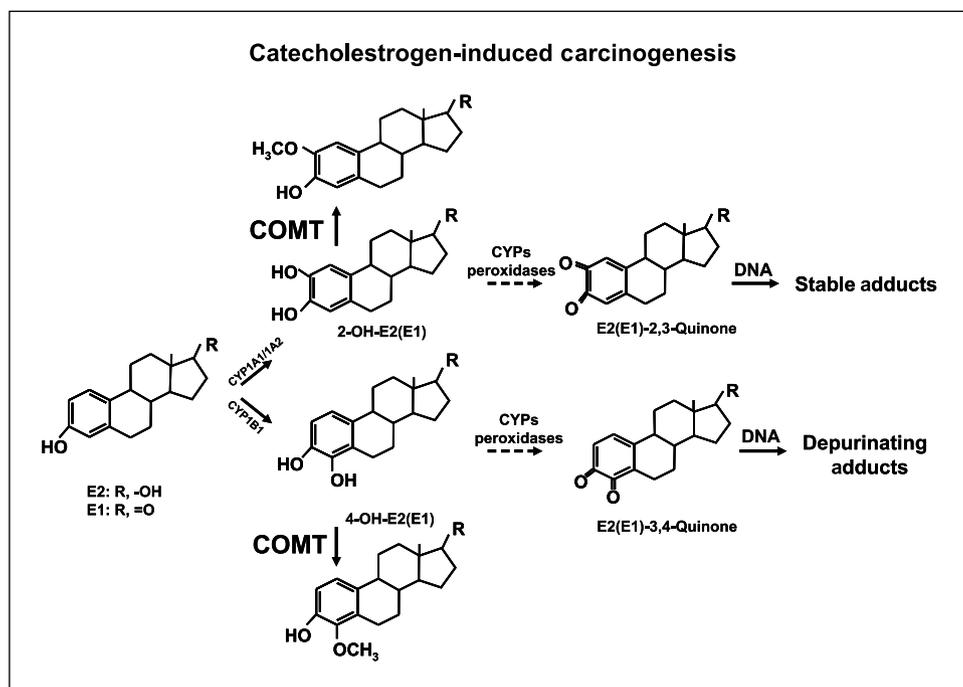
**Figure 1.** Catecholestrogen-induced carcinogenesis.

breast cancer risk. We began by resequencing the gene to determine the spectrum of common variation in *COMT* (Fig. 2A). Fifteen *COMT* polymorphisms were then chosen to genotype DNA samples from a large Mayo Clinic breast cancer case control study—followed by functional studies of polymorphisms that appeared to be associated with risk. We then performed two separate replication studies to test the observed association. This comprehensive approach resulted in the identification of two polymorphisms in the distal promoter of *COMT* that were associated with decreased breast cancer risk in two of the three studies—an association strongly supported by the functional genomic results—suggesting that MB-COMT might play a role in catecholestrogen metabolism in breast cancer. Therefore, our study strongly suggests that these MB-COMT single nucleotide polymorphisms (SNPs) may represent another of an increasing number of common polymorphisms that influence breast cancer risk (25).

## Materials and Methods

### SNP Selection for Genotyping

*COMT* was resequenced using DNA from 60 Caucasian-American subjects for all exons, splice-junctions, intron 2 (21), and 1 kb on either side of exon 1.[11] The *COMT* consensus sequence used in our studies was that of contig NT_011519.10. Numbering of nucleotides within the coding region began at the "A" in the "ATG" for MB-COMT, with nucleotides 3′ to that position assigned positive numbers. Nucleotides located within the two upstream noncoding exons, and the 5′-FR were assigned negative numbers, with the final nucleotide of exon 2 designed "−1" (i.e., the nucleotide 5′ of the ATG start codon). Nucleotides located within introns were numbered on the basis of distance from the nearest splice site, using positive and negative numbers for distance to 5′- and 3′-splice sites, respectively. The

numbering scheme for amino acids was based on their distance from the MB-COMT translation start codon. SNPs included in the genotyping studies were selected either by use of haplotype tagging (26) or the linkage disequilibrium (LD) select method (27). Fifteen SNPs were selected in this fashion or on the basis of previous reports of their association with clinical phenotypes (28, 29).

### Study Populations

**Mayo Clinic breast cancer case control study.** The Mayo Clinic study is an unselected, clinic-based series of breast cancer cases and healthy controls recruited in the Mayo Clinic Department of Oncology (30). Cases had a diagnosis of histologically confirmed primary breast cancer within 6 mo of enrollment and no prior history of cancer (except for nonmelanoma skin cancer). Controls were frequency matched to cases on county of residence and 5-y age group and were selected from women visiting the Mayo Clinic for a general medical examination. "Postmenopausal" was defined as having no menstrual period for 12 mo or having had the uterus and/or ovaries removed. Family history of cancer was reported for all first and second-degree relatives. Controls were ineligible if they had a previous diagnosis of cancer (except nonmelanoma skin cancer). As of June 2005, when the present study began, 750 cases and 732 controls, entirely Caucasian, had been recruited from the states of Iowa, Minnesota, or Wisconsin. Participation rates were 70% among cases and 72% among controls. All eligible women were asked to provide written informed consent, risk factor information via a written questionnaire, and a sample of blood. The study was reviewed and approved by the Mayo Clinic Institutional Review Board.

**The interdisciplinary study group on gene environment interactions and breast cancer in Germany study population (GENICA Study).** The GENICA study is a study of breast cancer cases and controls from the greater Bonn area in Germany (31). Specifically, between August 2000 and September 2004, 1,143 breast cancer cases and 1,155 population-based controls were recruited. Cases had a first-time diagnosis of primary breast cancer that was histologically confirmed within 6 mo of enrollment. Controls were population based and matched to cases in 5-y age groups. All participants were Caucasian and were ages <80 years. Risk factor information was collected via in-person interviews using the core questionnaire of a German population survey that had been extended by the inclusion of questions on factors related to breast cancer. The response

rates were 86% for cases and 69% for controls. A participant was classified as premenopausal if she reported menstrual periods in the year of interview. All participants provided a blood sample, and DNA was available for 1,015 cases and 1,021 controls. The GENICA study was reviewed and approved by the Ethics Committee of the University of Bonn, and all study participants provided written informed consent.

**The genetic epidemiology study of breast cancer by age 50 (GESBC).** The GESBC is a population-based case control study carried out in two geographic areas of Germany (Freiburg and the Rhein-Neckar-Odenwald regions) that included patients newly diagnosed with breast cancer between 1992 and 1995 (32). Subjects were German speaking (97% had 2 and 3% had 1 German parent), under age 51 years, and they could have no previous history of breast cancer. Cases had a confirmed diagnosis of either *in situ* or invasive breast cancer and were identified by monitoring ~40 hospitals. Participation was 70% among the 1,005 eligible and living cases. An attempt was made to recruit two population controls per case, matched by age and study region. Subjects were not eligible to be controls if they could not

speak German, had moved out of the study region, or had a previous history of primary breast cancer. Participation was 61% among the 2,257 eligible population controls. Subjects were defined as postmenopausal if they reported a natural menopause 6 mo before the reference date (date of diagnosis or date of questionnaire for control) or a bilateral oophorectomy. The menopausal status of women who reported hysterectomy alone was classified as unknown. Subjects were asked to complete a self-administered questionnaire on demographic and breast cancer risk factors, and to provide a blood sample. The GESBC study was reviewed and approved by the Ethics Committee of the University of Heidelberg, and subjects who participated provided written informed consent.

## Genotyping Methods

Fifteen *COMT* polymorphisms were used to genotype samples from the Mayo Clinic study. Most genotyping was performed with the SNPstream platform (Beckman Coulter). ABI fragment analysis assay was used to genotype the insertion/deletion polymorphism in the *COMT* 3′ untranslated
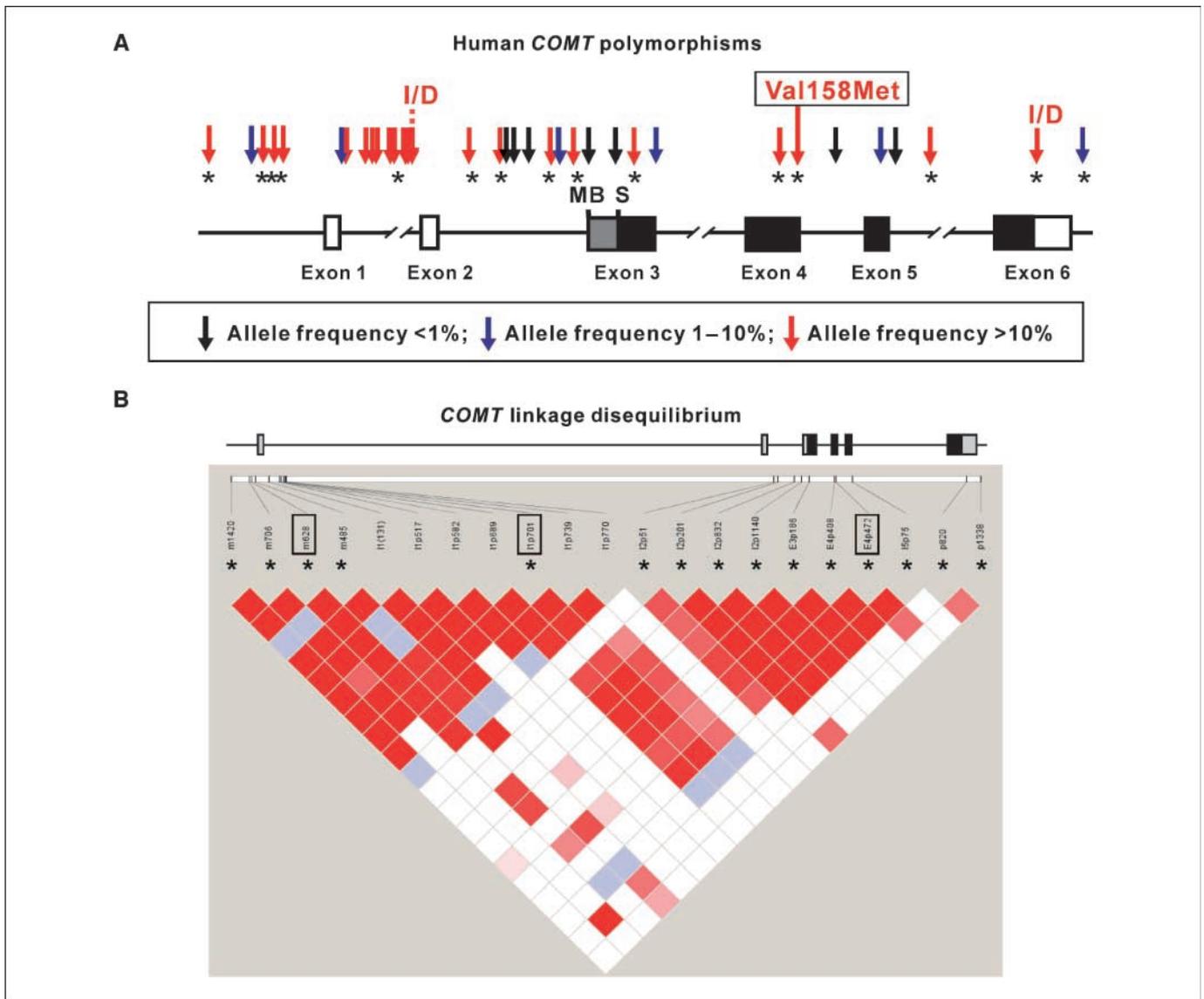


**Figure 2.** Human *COMT* genetic polymorphisms and linkage disequilibrium. *A, COMT* genetic polymorphisms in 60 Caucasian-American subjects. *Arrows,* locations of polymorphisms. *Black and gray rectangles,* coding exons with the gray area specific for the MB-COMT open reading frame (ORF). *Open rectangles,* noncoding exons. *I/D,* insertion/deletion. Alterations in amino acid sequence resulting from nonsynonymous SNPs are boxed. *, polymorphisms that were genotyped in the Mayo Clinic study. *B, COMT* linkage disequilibrium displayed by the use of Haploview 3.3. Polymorphisms with minor allele frequencies of <5% were excluded from the analysis. The coloring scheme is the standard Haploview (D′/LOD).

region (UTR; 820), and DNA sequencing was used to genotype five SNPs: 5′-FR(−1420), 5′-FR(−628), 5′-FR(−485), I2(832), and I2(1140). During the two replication studies, only 3 SNPs, 5′-FR(−628), I1(701), and the exon 4(472) Val108/158Met polymorphism, were genotyped. The GENICA samples were genotyped using Sequenom MALDI-TOF MS (33). Taq Man technology was used to genotype the GESBC samples (34).

**Statistical analysis.** Demographic information for cases and controls was summarized using means and SDs, or counts and percentages, as appropriate. Comparisons were made between case and control groups using $t$ tests or $\chi^2$ tests. Genotypes for each of the polymorphisms were used to estimate allele frequencies separately for cases and controls. Genotypes in the controls were also assessed for departures from Hardy-Weinberg equilibrium.

For the Mayo Clinic study, the association of each SNP genotype with case/control status was performed separately for premenopausal and postmenopausal strata. Within each stratum, unconditional logistic regression was used to evaluate the log-additive effects of the rare allele for each SNP, while adjusting for age and geographic region of residence. The log-additive effects were summarized by odds ratios (OR) and 95% confidence intervals (95% CI).

In addition to the matching variables, the effects of other covariates were evaluated using backward stepwise regression, and the additional significant covariates ($P < 0.05$) were parity/age at first child birth and physical activity. These additional covariates were also used for statistical adjustments in the logistic regression analyses. Although there have been a large number of studies of the effect of *COMT* on breast cancer risk, making the need to control for multiple testing for our study debatable, we nonetheless present $P$ values adjusted for multiple testing of 30 tests (15 SNPs and 2 menopausal strata). To adjust $P$ values, we randomly permuted case/control status within each menopausal status stratum 10,000 times to determine the distribution of the test statistics. The frequency that the largest permuted statistic (of 30) was greater than each of the observed statistics provided an empirical $P$ value adjusted for the 30 tests performed. Supplementary analyses were used to evaluate the association of SNP genotypes with ER status by directly comparing the ER(+) cases versus the ER(−) cases, once again using logistic regression with covariates.

For the GENICA and GESBC studies, backward covariate selection was used to choose the covariates HRT and physical activity for the GENICA study and physical activity and parity/age at first birth for the GESBC study. To perform pooled analyses across all three studies, relevant covariates were included as fixed effects and the study was included as a random effect, fitting the models with the SAS NLMIXED procedure. Analyses were performed in the SAS (SAS Institute), S-Plus (Insightful), and Haploview 3.3 (Daly Laboratory at the Broad Institute) programs.

### Functional Genomic Analyses

**Electromobility shift assays.** Electromobility shift (EMS) assays were performed with MCF-7 cell nuclear extract for oligonucleotides containing the wild-type (WT) or variant nucleotides (see Supplementary Table S1 for oligonucleotide sequences). The procedure has been described elsewhere (35). $^{32}$P-labeled annealed oligonucleotides were incubated with 5-μg nuclear extract protein (Active Motif) and 5X binding buffer (Promega Corporation) for the binding reactions. Nuclear extract was eliminated from the control reaction. One hundred-fold excess unlabeled specific oligonucleotide was added to the competition reactions. All reactions were incubated at room temperature for 20 min and were then subjected to PAGE. The AliBaba2.1[12] program predicted that the 5′-FR(−628) sequence might bind to AP2α. Therefore, a supershift assay was also performed with the WT C(−628) oligonucleotides using a rabbit polyclonal antibody against human AP2α (Santa Cruz Biotechnology, Inc.).

**Reporter gene studies.** The C(−628)T and I1A(701)G SNPs were located within an area containing the *COMT* distal promoter. To study their possible effect on transcription, reporter gene constructs were created by

cloning 1.5 kb of DNA sequence spanning the two SNPs into pGL3-Basic (Promega). Two single variants, as well as a double variant with both SNPs, were created from the WT construct by site-directed mutagenesis. Inserts were sequenced in both directions to ensure that the correct sequence was present. These reporter gene constructs were designated as the WT, (−628)C/I1(701)A, (−628)T/I1(701)A, (−628)C/I1(701)G, and (−628)T/I1(701)G—the double variant. Reporter gene constructs (1.5 μg of each reporter gene) were cotransfected with 20 ng of the pRL-TK vector into breast carcinoma MCF-7 cells, MDA468 cells, and hepatic carcinoma HepG2 cells, followed by dual luciferase assay (Promega). The values for relative luciferase activity were reported as percentages of the WT activity.

**Recombinant COMT expression and enzyme assays.** Expression constructs encoding the WT MB-COMT and S-COMT proteins were generated by cloning the cDNAs into the eukaryotic expression vector pCR3.1 (Invitrogen). These expression constructs were transfected into COS-1 cells and cytosol (containing S-COMT) and microsome (containing MB-COMT) preparations were isolated (36). COMT activity was measured with 4-hydroxyestradiol and 2-hydroxyestradiol (Sigma-Aldrich) as methyl acceptor catechol substrates (37). Apparent $K_m$ values for 4-OH-E2 and 2-OH-E2 were determined with a series of substrate concentrations ranging from 0.05 to 20 μmol/L.

**COMT exon array analyses.** Total RNA was extracted from flash-frozen breast cancer tissue from seven individual patients. All RNA samples had an Agilent RNA Integrity Number of >7.8. The RNA was then reverse transcribed and biotin labeled for hybridization with GeneChip Human Exon 1.0 ST Arrays (Affymetrix). Arrays were normalized with full quantile normalization using XRAY software (Biotique Systems, Inc.) and were background corrected using GC content–matched antigenomic probes by the application of median-polish (exon RMA). Log$_2$-transformed expression values were derived for each of the *COMT* "core" probe sets.

## Results

### *COMT* Haplotype Structure

*COMT* was resequenced using DNA samples from 60 Caucasian subjects (21). A total of 33 polymorphisms were observed, including the Val108/158Met polymorphism that has been studied so often (Fig. 2*A*). One third of the polymorphisms were located within the region flanking exon 1, an area that includes the distal promoter for MB-COMT. There seemed to be at least two clearly defined "haplotype blocks" in DNA samples from CA subjects (Fig. 2*B*).

### Mayo Clinic Breast Cancer Case Control Association Study

The Mayo Clinic study included 750 cases as well as 732 controls. Characteristics of these subjects, as well as the GENICA and GESBC subjects, are listed in Supplementary Table S2. For the Mayo Clinic study, the controls tended to have significantly more live births than did the cases, and fewer cases were highly physically active when compared with controls. For the GENICA study, cases also tended to be less active than controls, but not in the GESBC study, which recruited younger cases. Finally, cases had a higher frequency of family history of breast or ovarian cancer than did their controls in all three studies. All SNP genotypes fit HWE proportions among the controls ($P$ values ranging from 0.14–0.94 across all three groups), indicating high-quality genotyping.

Associations of *COMT* SNPs with case/control status for the Mayo Clinic study are summarized in Table 1. Two SNPs, rs2020917[C(−628)T] and rs737865 [I1A(701)G], seemed to be associated with breast cancer risk reduction in premenopausal women. The analysis was stratified for menopausal status because previous studies of *COMT* and breast cancer risk had shown differences between premenopausal and postmenopausal women (22, 24), and because of a great deal of evidence that the

---

[12] http://www.gene-regulation.com/pub/programs/alibaba2/index.html

pathophysiology of the disease may differ in these two groups—including recent data from genome-wide association studies (38–40). The estimated allele-specific ORs for the rare alleles of both SNPs were ~0.70, and adjustment for covariates had little effect on these estimates. Comparing the log-additive model of allele effects to the general model for codominant effects did not show any statistically significant departures from the log-additive effects. Additional analyses (not presented) suggested that the effects of these two SNPs did not differ significantly between ER(+) and ER(−) cases within each of the menopausal strata (P values ranging from 0.28–0.68). These two SNPs were in high LD, both in breast cancer cases (D′ = 0.98 and $R^2$ = 0.95) and controls (D = 0.91 and $R^2$ = 0.78). Although the P values unadjusted for multiple testing suggested that these SNPs provide a reduced risk of breast cancer among premenopausal women [P = 0.022 for 5′-FR(−628) and P = 0.011 for I1(701)], after correcting for multiple testing, the associations no longer met the usual criteria for statistical

significance (P = 0.201 and 0.102, respectively). However, given the number of studies that have reported a positive association of *COMT* with breast cancer, we concluded that these two SNPs were worth pursuing in replicate studies, especially considering the results of the functional genomic experiments described subsequently.

The Val108/158Met polymorphism at exon 4(472) that has been studied in all previous *COMT* breast cancer studies did not seem to be associated with breast cancer risk in the Mayo study. Both of the distal promoter SNPs, 5′-FR(−628) and I1(701), were moderately linked to the Val108/158Met polymorphism (D′ = 0.78, $R^2$ = 0.21 and D′ = 0.91, $R^2$ = 0.31, respectively) in the samples used for our *COMT* resequencing studies.

## Functional Genomic Studies

**EMS assays and reporter gene studies.** The results of the Mayo Clinic study suggested that the two distal promoter SNPs, 5′-FR(−628) and I1(701), might play a role in breast cancer risk

**Table 1.** Association of SNPs for Mayo Clinic breast cancer cases and controls, stratified on menopausal status

| Menopausal Status | SNP | rs# | MAF | | Adjusted-Matching* | | | Permutation | Adjusted covariates [†] | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | Cases | Controls | OR (allelic) | 95% CI | P | P | OR (allelic) | 95% CI | P |
| Premenopausal | | | n = 264 | n = 193 | | | | | | | |
| | 5′FR(−1420) | rs2075507 | 0.46 | 0.43 | 1.07 | 0.81–1.41 | 0.619 | 0.999 | 1.07 | 0.81–1.42 | 0.635 |
| | 5′FR(−706) | rs4646310 | 0.19 | 0.18 | 1.03 | 0.72–1.47 | 0.869 | 1.000 | 1.05 | 0.73–1.50 | 0.791 |
| | 5′FR(−628) | rs2020917 | 0.22 | 0.30 | 0.70 | 0.52–0.95 | 0.022 | 0.201 | 0.70 | 0.52–0.96 | 0.024 |
| | 5′FR(−485) | rs13306278 | 0.11 | 0.14 | 0.79 | 0.53–1.17 | 0.241 | 0.966 | 0.77 | 0.52–1.16 | 0.217 |
| | Intron1(701) | rs737865 | 0.23 | 0.32 | 0.68 | 0.51–0.92 | 0.011 | 0.102 | 0.69 | 0.51–0.92 | 0.013 |
| | Intron2(51) | rs165656 | 0.44 | 0.48 | 0.86 | 0.66–1.13 | 0.274 | 0.975 | 0.88 | 0.67–1.16 | 0.379 |
| | Intron2(201) | rs165722 | 0.44 | 0.48 | 0.87 | 0.67–1.14 | 0.316 | 0.985 | 0.90 | 0.69–1.18 | 0.453 |
| | Intron2(832) | rs3810595 | 0.35 | 0.41 | 0.79 | 0.60–1.03 | 0.080 | 0.662 | 0.81 | 0.61–1.06 | 0.130 |
| | Intron2(1140) | rs6269 | 0.36 | 0.42 | 0.78 | 0.60–1.02 | 0.072 | 0.666 | 0.82 | 0.62–1.08 | 0.153 |
| | Exon3(186) | rs4633 | 0.44 | 0.48 | 0.88 | 0.68–1.15 | 0.358 | 0.991 | 0.91 | 0.70–1.20 | 0.509 |
| | Exon4(408) | rs4818 | 0.34 | 0.40 | 0.78 | 0.59–1.03 | 0.081 | 0.703 | 0.81 | 0.61–1.07 | 0.134 |
| | Exon4(472) | rs4680 | 0.42 | 0.46 | 0.89 | 0.68–1.17 | 0.411 | 0.992 | 0.91 | 0.69–1.20 | 0.499 |
| | Intron5(75) | rs4646315 | 0.23 | 0.19 | 1.18 | 0.84–1.67 | 0.336 | 0.795 | 1.14 | 0.81–1.62 | 0.454 |
| | 3′UTR(820 indel) | rs362204 | 0.24 | 0.25 | 0.97 | 0.69–1.37 | 0.882 | 1.000 | 0.99 | 0.70–1.41 | 0.976 |
| | 3′FR(1338) | rs165599 | 0.29 | 0.30 | 0.98 | 0.73–1.32 | 0.884 | 1.000 | 0.99 | 0.73–1.35 | 0.972 |
| Postmenopausal | | | n = 454 | n = 521 | | | | | | | |
| | 5′FR(−1420) | | 0.43 | 0.43 | 1.01 | 0.83–1.23 | 0.917 | 1.000 | 0.98 | 0.81–1.20 | 0.853 |
| | 5′FR(−706) | | 0.18 | 0.17 | 1.05 | 0.82–1.35 | 0.714 | 1.000 | 1.07 | 0.83–1.39 | 0.594 |
| | 5′FR(−628) | | 0.26 | 0.27 | 0.94 | 0.76–1.17 | 0.600 | 1.000 | 0.97 | 0.78–1.20 | 0.765 |
| | 5′FR(−485) | | 0.14 | 0.15 | 0.89 | 0.67–1.17 | 0.389 | 1.000 | 0.90 | 0.68–1.18 | 0.436 |
| | Intron1(701) | | 0.27 | 0.27 | 0.97 | 0.78–1.21 | 0.795 | 1.000 | 1.00 | 0.80–1.25 | 0.991 |
| | Intron2(51) | | 0.47 | 0.48 | 0.97 | 0.81–1.17 | 0.765 | 1.00 | 0.98 | 0.81–1.19 | 0.863 |
| | Intron2(201) | | 0.47 | 0.48 | 0.94 | 0.79–1.13 | 0.524 | 1.000 | 0.96 | 0.79–1.15 | 0.643 |
| | Intron2(832) | | 0.37 | 0.40 | 0.89 | 0.74–1.07 | 0.210 | 0.962 | 0.90 | 0.74–1.09 | 0.279 |
| | Intron2(1140) | | 0.37 | 0.41 | 0.84 | 0.70–1.02 | 0.073 | 0.657 | 0.86 | 0.71–1.04 | 0.109 |
| | Exon3(186) | | 0.47 | 0.49 | 0.92 | 0.77–1.11 | 0.383 | 1.000 | 0.93 | 0.77–1.12 | 0.444 |
| | Exon4(408) | | 0.36 | 0.39 | 0.90 | 0.75–1.09 | 0.296 | 1.000 | 0.91 | 0.75–1.11 | 0.364 |
| | Exon4(472) | | 0.46 | 0.47 | 0.95 | 0.78–1.14 | 0.567 | 1.000 | 0.95 | 0.78–1.15 | 0.608 |
| | Intron5(75) | | 0.20 | 0.18 | 1.15 | 0.90–1.46 | 0.263 | 0.975 | 1.14 | 0.89–1.46 | 0.300 |
| | 3′UTR(820indel) | | 0.26 | 0.27 | 0.95 | 0.76–1.18 | 0.619 | 1.000 | 0.95 | 0.76–1.18 | 0.626 |
| | 3′FR(1338) | | 0.32 | 0.30 | 1.03 | 0.84–1.26 | 0.806 | 1.000 | 1.04 | 0.84–1.28 | 0.742 |

NOTE: The three SNPs that were genotyped in the two replication studies are "boxed".
Abbreviations: MAF, minor allele frequency.
*Adjusted for matching variables of age and area.
[†]Adjusted for age, area, parity/age at first child birth, and physical activity.
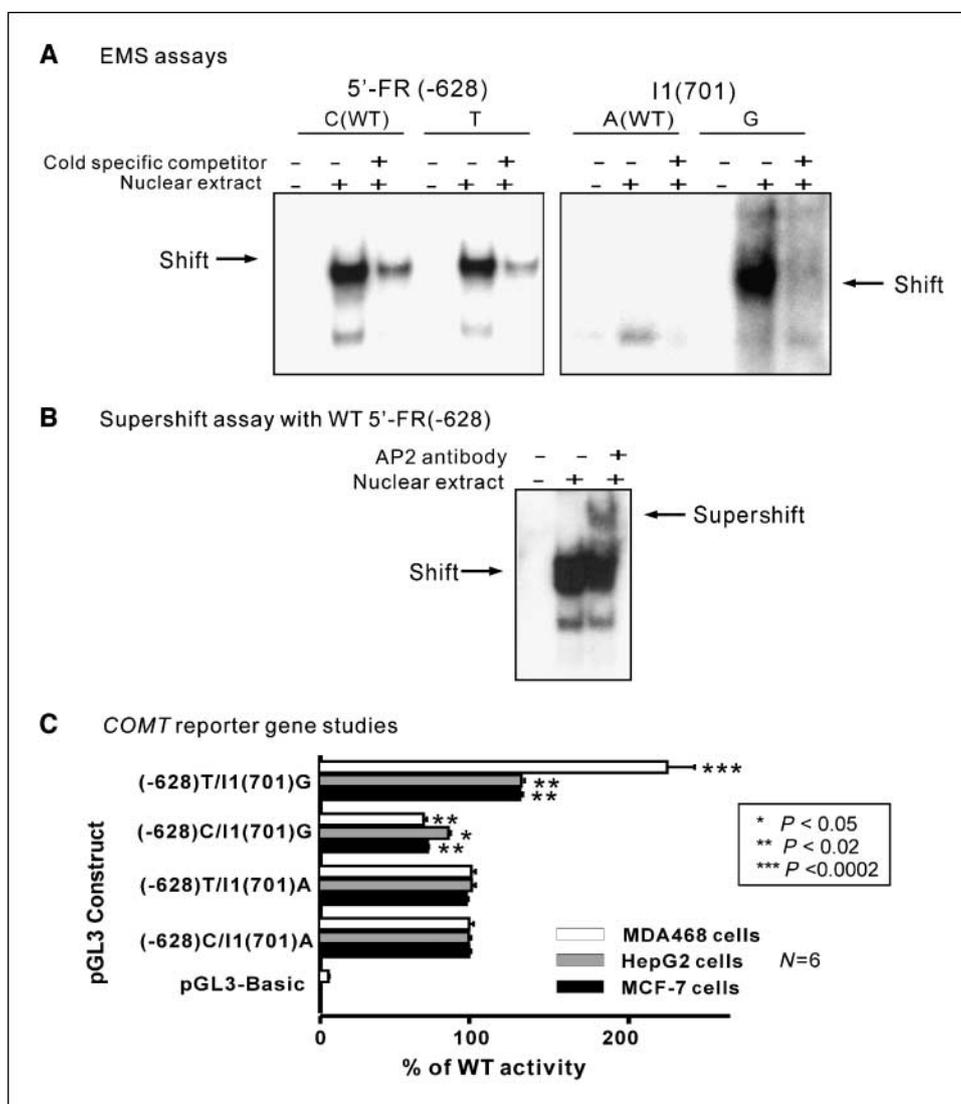
**Figure 3.** COMT functional genomic studies. *A,* EMS assays for 5'-FR(−628) and I1(701) SNPs. MCF-7 cell nuclear extract was incubated with $^{32}$P-labled oligonucleotides. *B,* supershift assay for WT 5'-FR(−628). A rabbit polyclonal antibody against the human AP-2α was incubated with $^{32}$P-labled WT. 5'-FR (−628) oligonucleotides and MCF-7 cell nuclear extract. *C,* reporter gene studies. Activities of luciferase reporter gene constructs containing different combinations of the two loci, 5'-FR(−628) and I1(701), are shown as a percentage of the level of relative luciferase activity for the WT construct, (−628)C/I1(701)A. *Columns,* mean of six independent transfections; *bars,* SE.

reduction. However, before attempting replication, we performed a series of functional genomic experiments to determine the biological plausibility of these SNPs. First, EMS assays were performed with MCF-7 cell nuclear extract. Figure 3*A* shows that nuclear protein(s) bound to sequences at or near nucleotide (−628), but the change from C to T at that position did not alter the binding pattern. On the basis of a supershift assay, the protein bound to the (−628) locus might be an AP2 transcription factor (Fig. 3*B*). At the I1(701) locus, WT oligonucleotides did not bind protein, but protein binding occurred with the variant oligonucleotide sequence (Fig. 3*A*).

Next, reporter gene studies were performed. Constructs for single variants with each SNP alone as well as a construct containing both variant nucleotides were created using site-directed mutagenesis. The double variant had the greatest effect on transcription when compared with the WT in all three cell lines studied (all $P < 0.002$; Fig. 3*C*). This effect was particularly striking in MDA468 cells, with an average 2.3-fold increase ($P < 0.0002$).

**Substrate kinetic and exon array analyses.** Both SNPs that seemed to influence breast cancer risk were located in the area containing the promoter for MB-COMT. Although it is known that

MB-COMT catalyzes the methylation of catecholamine neurotransmitters with lower $K_m$ values than those for S-COMT (41), there is no similar evidence for catecholestrogens, so we performed catecholestrogen substrate kinetic studies. Both 2-OH and 4-OH catecholestrogens were substrates for *O*-methylation catalyzed by MB-COMT (Fig. 4*A*). The apparent $K_m$ for S-COMT was $16.1 \pm 2.0$ μmol/L (mean ± SE) for 4-OH-E2 and $9.0 \pm 0.66$ μmol/L for 2-OH-E2. MB-COMT had significantly lower $K_m$ values for both catecholestrogen substrates: $6.1 \pm 0.55$ μmol/L for 4-OH-E2 and $1.0 \pm 0.04$ μmol/L for 2-OH-E2 ($P < 0.05$ compared with $K_m$ values for S-COMT). We also performed exon array analyses with RNA from breast cancer tissue to determine whether MB-COMT was expressed in that tissue. Exon array analyses performed with total RNA extracted from seven breast cancer tissue samples (Fig. 4*B*) showed that the two noncoding upstream exons, exons present only in MB-COMT mRNA, were expressed in all breast tumor samples.

Results from these functional genomic studies supported a role for MB-COMT and the two distal promoter SNPs identified during the Mayo Clinic study. This evidence of biological plausibility provided a stimulus for the replication studies described subsequently.

### Replication Studies

The two replication studies performed in collaboration with GENICA and GESBC involved genotyping only three SNPs (thus reducing multiple testing), the heavily studied Val108/158Met—exon 4(472) polymorphism and the two distal promoter SNPs. The association of these SNPs with case control status in the GENICA and GESBC samples is illustrated in Table 2. For the GENICA study, the 5′-FR(−628) and I1(701) SNPs were significantly associated with a reduced risk for breast cancer in the postmenopausal group. Although the associations of these SNPs in the premenopausal stratum were not statistically significant by the usual criterion of $P$ value of <0.05, the sample sizes were much smaller for the premenopausal stratum, and the magnitude of the allelic effects for the GENICA study were consistent between the premenopausal and postmenopausal strata, as well as with the risk reduction observed in the Mayo Clinic premenopausal stratum. In contrast, the GESBC study did not show a risk reduction for these SNPs. In fact, the ORs for that study were slightly >1.0.

Finally, in an attempt to evaluate effect sizes pooled across all three studies, we allowed the covariates to differ for the three studies (each study was adjusted for its own set of covariates) but estimated a pooled effect size of the allelic OR for each of the SNPs (see Supplementary Table S3). Two different $P$ values are listed as follows: the $P$ value for OR = 1 tests whether the pooled OR differs significantly from 1.0, whereas the $P$ value for heterogeneity tests whether the ORs differ across the three studies. The ORs for 5′-FR(−628) and I1(701) differed significantly among the three studies for the premenopausal stratum. Tables 1 and 2 show that these SNPs were associated with reduced risk for both the Mayo Clinic and GENICA studies in at least one of the menopausal cohorts but displayed no effect in either cohort for the GESBC study. In contrast, none of the ORs differed significantly in the postmenopausal stratum.

## Discussion

Known breast cancer susceptibility genes, including *BRCA1* and *BRCA2*, account for only a small portion of the familial risk for breast cancer, resulting in many studies with the goal of identifying low-penetrance risk genes (42). However, most of those efforts were unsuccessful before a recent series of large genome-wide association studies (25, 39, 40). Those studies identified common polymorphisms that might represent novel markers for breast cancer risk. The largest of those studies concluded that many additional common susceptibility alleles remained to be identified (25). Estrogen exposure is an established breast cancer risk factor (2, 3). COMT could potentially reduce risk for estrogen-dependent carcinogenesis in both ER-dependent and non–ER-dependent pathways by catalyzing the $O$-methylation of catecholestrogens (Fig. 1; refs. 5, 7). As a result, numerous breast cancer association studies have been performed, almost all focusing on the Val108/158Met polymorphism (22–24), and the results of those studies have been inconsistent.



**Figure 4.** COMT isoform–specific substrate kinetics and expression in breast cancer tissue. *A,* substrate curves for the methylation of 4-OH-E2 and 2-OH-E2 catalyzed by either MB-COMT or S-COMT. *Points,* mean for three determinations; *bars,* SE. *B,* exon array analysis of COMT in human breast cancer tissue. Each point represents the normalized expression value for that probe set. The *COMT* gene structure is also shown; *blue boxes,* ORF; *gray boxes,* noncoding exons.
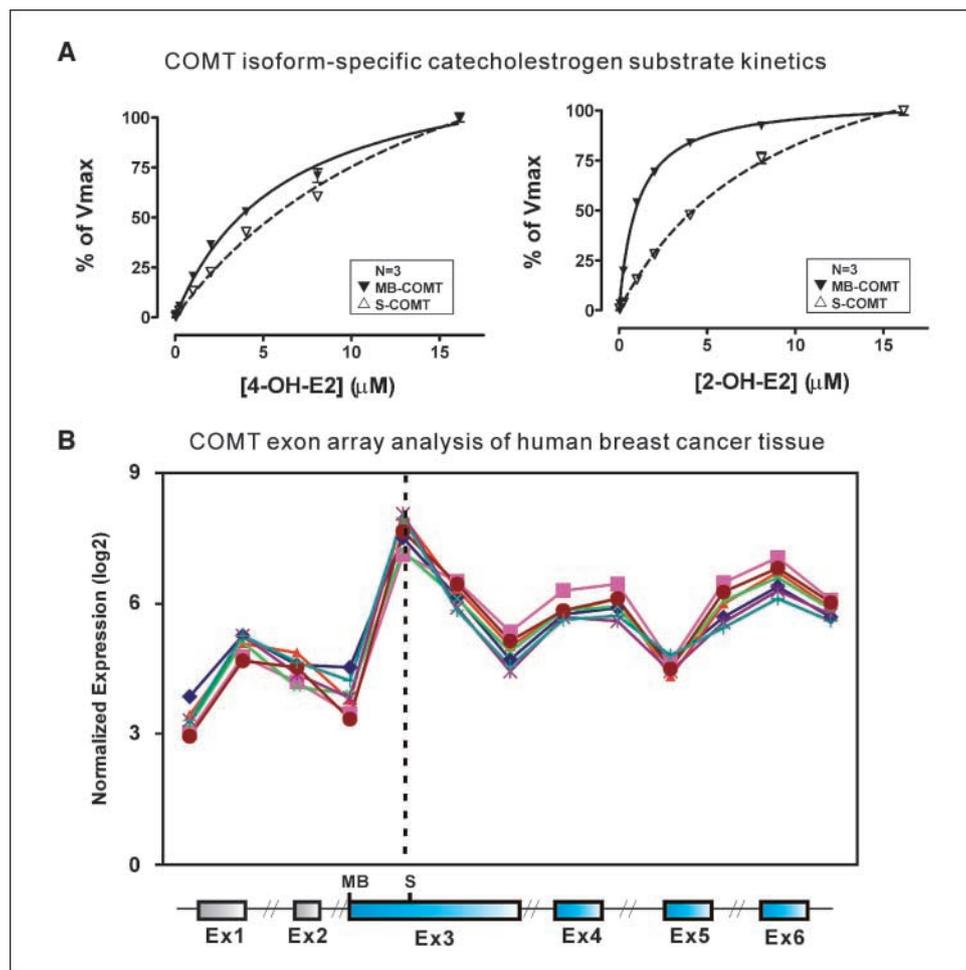
**Table 2.** Association of SNPs in the two replication studies, stratified on menopausal status

| Group | Menopausal Status | SNP | MAF | | Unadjusted | | | Adjusted for covariates | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | Cases | Controls | OR (allelic) | 95% CI | P | OR (allelic) | 95% CI | P |
| GENICA | Premenopausal | | n = 249 | n = 234 | | | | * | | |
| | | 5'FR(−628) | 0.26 | 0.30 | 0.79 | 0.59–1.05 | 0.106 | 0.78 | 0.59–1.05 | 0.104 |
| | | Intron1(701) | 0.24 | 0.28 | 0.79 | 0.59–1.06 | 0.114 | 0.78 | 0.58–1.05 | 0.106 |
| | | Exon4(472) | 0.47 | 0.48 | 0.98 | 0.76–1.26 | 0.884 | 0.98 | 0.76–1.26 | 0.867 |
| | Postmenopausal | | n = 758 | n = 763 | | | | * | | |
| | | 5'FR(−628) | 0.25 | 0.28 | 0.85 | 0.72–1.00 | 0.044 | 0.85 | 0.72–1.00 | 0.053 |
| | | Intron1(701) | 0.23 | 0.26 | 0.84 | 0.72–0.99 | 0.042 | 0.85 | 0.72–1.01 | 0.061 |
| | | Exon4(472) | 0.49 | 0.47 | 1.07 | 0.93–1.23 | 0.351 | 1.07 | 0.93–1.23 | 0.367 |
| GESBC | Premenopausal | | n = 438 | n = 877 | | | | † | | |
| | | 5'FR(−628) | 0.30 | 0.28 | 1.08 | 0.90–1.28 | 0.412 | 1.09 | 0.91–1.31 | 0.337 |
| | | Intron1(701) | 0.30 | 0.29 | 1.04 | 0.87–1.24 | 0.655 | 1.05 | 0.88–1.26 | 0.587 |
| | | Exon4(472) | 0.50 | 0.49 | 1.03 | 0.88–1.21 | 0.719 | 1.03 | 0.87–1.22 | 0.740 |
| | Postmenopausal | | n = 124 | n = 208 | | | | † | | |
| | | 5'FR(−628) | 0.30 | 0.29 | 1.05 | 0.74–1.49 | 0.783 | 1.06 | 0.74–1.51 | 0.743 |
| | | Intron1(701) | 0.30 | 0.29 | 1.04 | 0.73–1.47 | 0.835 | 1.04 | 0.73–1.48 | 0.827 |
| | | Exon4(472) | 0.46 | 0.49 | 0.87 | 0.63–1.20 | 0.399 | 0.87 | 0.63–1.21 | 0.400 |

*Adjusted for HRT use and physical activity.
†Adjusted for physical activity and parity/age at first child birth.

In the present study, we took a comprehensive approach that began with resequencing of the *COMT* gene (Fig. 2*A*). Fifteen of the 33 polymorphisms identified in DNA from Caucasian subjects were used to genotype DNA samples from a large Mayo Clinic breast cancer case control study. Those 15 polymorphisms were scattered across the entire length of the gene within both major haplotype blocks present in Caucasian subjects (Fig. 2*B*). We observed a reduction in breast cancer risk in premenopausal women associated with two SNPs in the "distal promoter" for MB-COMT, but the heavily studied Val108/158Met polymorphism failed to show a significant association with risk.

Functional genomic studies of these two polymorphisms showed that they altered nuclear protein binding patterns and were associated with the up-regulation of transcription—potentially resulting in increased COMT activity—compatible with reduced risk if the "catecholestrogen carcinogenesis hypothesis" is correct. These two SNPs were highly linked, but both were only moderately linked to the Val108/158Met polymorphism, possibly helping to explain why that polymorphism has been inconsistently associated with risk. Figure 2*B* shows at least two large haplotype blocks in *COMT*, and 5′-FR(−628) and I1(701) are both located in the upstream block associated with MB-COMT. The location of these two functional polymorphisms in the distal promoter suggested that MB-COMT might play a role in estrogen-induced carcinogenesis. Previous studies were based on the tacit assumption that S-COMT is the major isoform in most peripheral tissues, whereas MB isoform is most important in the brain (14, 15). However, our substrate kinetic experiments showed that MB-COMT has a higher affinity for catecholestrogens than does S-COMT (Fig. 4*A*). Furthermore, exon array analysis for seven breast cancer tissue samples indicated that MB-COMT is expressed in breast tumors, with expression that might exceed that of S-COMT, thus confirming a previous report that MB-COMT is expressed in breast cancer tissue (Fig. 4*B*; ref. 43).

We next extended our studies to include two independent breast cancer case control studies of similar ethnic composition. In the GENICA study, the protective effect of the two distal promoter SNPs was consistent with the Mayo results and that effect was observed for both the premenopausal and postmenopausal strata. However, the GESBC study, which consists mainly of premenopausal subjects, failed to replicate observations made for both Mayo Clinic and GENICA subjects. Heterogeneity of study results is a constant problem in association studies. However, our results showed that ORs differed significantly among the premenopausal women across all three studies. Differences in study design, composition of study populations, and underlying genetic and/or environmental factors might all contribute to these heterogeneous results. In addition, the possible effect of one or more undetected confounding factors in the GESBC study might have diluted the moderate protective role of these *COMT* variants. However, it should be emphasized that all of our functional observations were also compatible with a possible protective role for MB-COMT in estrogen-induced carcinogenesis. Subsequently, a complementary study was performed using the same Mayo Clinic study, and one of our two MB-COMT SNPs was also found to be associated with decreased mammographic density (44), further supporting our hypothesis because decreased mammographic density is associated with reduced breast cancer risk (45). These distal promoter SNPs should also be included in future neuropsychiatric studies of *COMT*.

In summary, we have identified two common, functionally significant polymorphisms located in the distal promoter of *COMT* which were associated with breast cancer risk reduction in 2 of 3 independent association studies—studies that included a total of 2,327 cases and 2,838 controls. Furthermore, laboratory-based functional genomic studies provided strong mechanistic support for these epidemiologic observations and suggested that the MB form of the enzyme might contribute to a possible protective role for COMT in estrogen-induced breast carcinogenesis.

## Disclosure of Potential Conflicts of Interest

## Acknowledgments

## References

1. Smigal C, Jemal A, Ward E, et al. Trends in breast cancer by race and ethnicity: update 2006. CA Cancer J Clin 2006;56:168–83.
2. Feigelson HS, Henderson BE. Estrogens and breast cancer. Carcinogenesis 1996;17:2279–84.
3. Madigan MP, Ziegler RG, Benichou J, Byrne C, Hoover RN. Proportion of breast cancer cases in the United States explained by well-established risk factors. J Natl Cancer Inst 1995;87:1681–5.
4. Henderson BE, Ross R, Bernstein L. Estrogens as a cause of human cancer: the Richard and Hinda Rosenthal Foundation award lecture. Cancer Res 1988; 48:246–53.
5. Cavalieri EL, Stack DE, Devanesan PD, et al. Molecular origin of cancer: catechol estrogen-3,4-quinones as endogenous tumor initiators. Proc Natl Acad Sci U S A 1997;94:10937–42.
6. Yager JD, Davidson NE. Estrogen carcinogenesis in breast cancer. N Engl J Med 2006;354:270–82.
7. Cavalieri E, Chakravarti D, Guttenplan J, et al. Catechol estrogen quinones as initiators of breast and other human cancers: implications for biomarkers of susceptibility and cancer prevention. Biochim Biophys Acta Reviews on Cancer 2006;1766:63–78.
8. Martucci C, Fishman J. Uterine estrogen receptor binding of catecholestrogens and of estetrol (1,3,5(10)-estratriene-3,15α,16α,17β-tetrol). Steroids 1976;27:325–33.
9. Van Aswegen CH, Purdy RH, Wittliff JL. Binding of 2-hydroxyestradiol and 4-hydroxyestradiol to estrogen receptors from human breast cancers. J Steroid Biochem 1989;32:485–92.
10. Paul SM, Axelrod J. A rapid and sensitive radioenzymatic assay for catechol estrogens in tissues. Life Sci 1977;21:493–502.
11. Hoffman AR, Paul SM, Axelrod J. Catecholestrogen synthesis and metabolism by human breast tumors in vitro. Cancer Res 1979;39:4584–7.
12. Zhao Z, Kosinska W, Khmelnitsky M, et al. Mutagenic activity of 4-hydroxyestradiol, but not 2-hydroxyestradiol, in BB rat2 embryonic cells, and the mutational spectrum of 4-hydroxyestradiol. Chem Res Toxicol 2006; 19:475–9.
13. Lundstrom K, Tenhunen J, Tilgmann C, Karhunen T, Panula P, Ulmanen I. Cloning, expression and structure of catechol-O-methyltransferase. Biochim Biophys Acta 1995;1251:1–10.
14. Tenhunen J, Salminen M, Lundstrom K, Kiviluoto T, Savolainen R, Ulmanen I. Genomic organization of the human catechol O-methyltransferase gene and its expression from two distinct promoters. Eur J Biochem 1994;223:1049–59.
15. Jeffery DR, Roth JA. Characterization of membrane-bound and soluble catechol-O-methyltransferase from human frontal cortex. J Neurochem 1984;42:826–32.
16. Weinshilboum RM, Raymond FA. Inheritance of low erythrocyte catechol-o-methyltransferase activity in man. Am J Hum Genet 1977;29:125–35.

17. Scanlon PD, Raymond FA, Weinshilboum RM. Catechol-O-methyltransferase: thermolabile enzyme in erythrocytes of subjects homozygous for allele for low activity. Science 1979;203:63–5.
18. Lachman HM, Papolos DF, Saito T, Yu YM, Szumlanski CL, Weinshilboum RM. Human catechol-O-methyltransferase pharmacogenetics: description of a functional polymorphism and its potential application to neuropsychiatric disorders. Pharmacogenetics 1996;6:243–50.
19. Grossman MH, Szumlanski C, Littrell JB, Weinstein R, Weinshilboum RM. Electrophoretic analysis of low and high activity forms of catechol-O-methyltransferase in human erythrocytes. Life Sci 1992;50:473–80.
20. Lotta T, Vidgren J, Tilgmann C, et al. Kinetics of human soluble and membrane-bound catechol O-methyltransferase: a revised mechanism and description of the thermolabile variant of the enzyme. Biochemistry 1995;34:4202–10.
21. Shield AJ, Thomae BA, Eckloff BW, Wieben ED, Weinshilboum RM. Human catechol O-methyltransferase genetic variation: gene resequencing and functional characterization of variant allozymes. Mol Psychiatry 2004;9:151–60.
22. Lavigne JA, Helzlsouer KJ, Huang HY, et al. An association between the allele coding for a low activity variant of catechol-O-methyltransferase and the risk for breast cancer. Cancer Res 1997;57:5493–7.
23. Millikan RC, Pittman GS, Tse CK, et al. Catechol-O-methyltransferase and breast cancer risk. Carcinogenesis 1998;19:1943–7.
24. Thompson PA, Shields PG, Freudenheim JL, et al. Genetic polymorphisms in catechol-O-methyltransferase, menopausal status, and breast cancer risk. Cancer Res 1998;58:2107–10.
25. Easton DF, Pooley KA, Dunning AM, et al. Genome-wide association study identifies novel breast cancer susceptibility loci. Nature 2007;447:1087–93.
26. Stram DO, Haiman CA, Hirschhorn JN, et al. Choosing haplotype-tagging SNPS based on unphased genotype data using a preliminary sample of unrelated subjects with an example from the Multiethnic Cohort Study. Hum Hered 2003;55:27–36.
27. Carlson CS, Eberle MA, Rieder MJ, Yi Q, Kruglyak L, Nickerson DA. Selecting a maximally informative set of single-nucleotide polymorphisms for association analyses using linkage disequilibrium. Am J Hum Genet 2004; 74:106–20.
28. Shifman S, Bronstein M, Sternfeld M, et al. A highly significant association between a COMT haplotype and schizophrenia. Am J Hum Genet 2002;71:1296–302.
29. Nackley AG, Shabalina SA, Tchivileva IE, et al. Human catechol-O-methyltransferase haplotypes modulate protein expression by altering mRNA secondary structure. Science 2006;314:1930–3.
30. Olson JE, Ingle JN, Ma CX, et al. A comprehensive examination of CYP19 variation and risk of breast cancer using two haplotype-tagging approaches. Breast Cancer Res Treat 2006;102:237–47. Epub 2006 Sep 27.
31. Justenhoven C, Hamann U, Pesch B, et al. ERCC2

genotypes and a corresponding haplotype are linked with breast cancer risk in a German population. Cancer Epidemiol Biomarkers Prev 2004;13:2059–64.
32. Chang-Claude J, Eby N, Kiechle M, Bastert G, Becher H. Breastfeeding and breast cancer risk by age 50 among women in Germany. Cancer Causes Control 2000;11: 687–95.
33. Justenhoven C, Hamann U, Schubert F, et al. Breast cancer: a candidate gene approach across the estrogen metabolic pathway. Breast Cancer Res Treat 2007;108: 137–49. Epub 2007 Jun 23.
34. Slanger TE, Chang-Claude J, Wang-Gohrke S. Manganese superoxide dismutase Ala-9Val polymorphism, environmental modifiers, and risk of breast cancer in a German population. Cancer Causes Control 2006;17: 1025–31.
35. Wang L, Thomae B, Eckloff B, Wieben E, Weinshilboum R. Human histamine N-methyltransferase pharmacogenetics: gene resequencing, promoter characterization, and functional studies of a common 5′-flanking region single nucleotide polymorphism (SNP). Biochem Pharmacol 2002;64:699–710.
36. Sladek-Chelgren S, Weinshilboum RM. Catechol-o-methyltransferase biochemical genetics: human lymphocyte enzyme. Biochem Genet 1981;19:1037–53.
37. Raymond FA, Weinshilboum RM. Microassay of human erythrocyte catechol-O-methyltransferase: removal of inhibitory calcium ion with chelating resin. Clin Chim Acta 1975;58:185–94.
38. Friedenreich CM. Physical activity and breast cancer risk: the effect of menopausal status. Exerc Sport Sci Rev 2004;32:180–4.
39. Hunter DJ, Kraft P, Jacobs KB, et al. A genome-wide association study identifies alleles in FGFR2 associated with risk of sporadic postmenopausal breast cancer. Nat Genet 2007;39:870–4.
40. Stacey SN, Manolescu A, Sulem P, et al. Common variants on chromosomes 2q35 and 16q12 confer susceptibility to estrogen receptor-positive breast cancer. Nat Genet 2007;39:865–9.
41. Mannisto PT, Kaakkola S. Catechol-O-methyltransferase (COMT): biochemistry, molecular biology, pharmacology, and clinical efficacy of the new selective COMT inhibitors. Pharmacol Rev 1999;51:593–628.
42. Pharoah PD, Antoniou A, Bobrow M, Zimmern RL, Easton DF, Ponder BA. Polygenic susceptibility to breast cancer and implications for prevention. Nat Genet 2002; 31:33–6.
43. Tenhunen J, Heikkila P, Alanko A, Heinonen E, Akkila J, Ulmanen I. Soluble and membrane-bound catechol-O-methyltransferase in normal and malignant mammary gland. Cancer Lett 1999;144:75–84.
44. Olson JE, Ji Y, Vachon CM, et al. Mammogrpahic breast density and membrane-bound catechol-O-methyltransferase (COMT) genetic polymorphisms [abstract 525]. Proc Am Assoc Cancer Res 2008;49:122.
45. McCormack VA, dos Santos Silva I. Breast density and parenchymal patterns as markers of breast cancer risk: a meta-analysis. Cancer Epidemiol Biomarkers Prev 2006;15:1159–69.

**AACR** American Association for Cancer Research

# Breast Cancer Risk Reduction and Membrane-Bound Catechol *O*-Methyltransferase Genetic Polymorphisms

Yuan Ji, Janet Olson, Jianping Zhang, et al.

| | |
|---|---|
| **Updated version** | Access the most recent version of this article at:<br>http://cancerres.aacrjournals.org/content/68/14/5997 |
| **Supplementary Material** | Access the most recent supplemental material at:<br>http://cancerres.aacrjournals.org/content/suppl/2008/07/09/68.14.5997.DC1 |

| | |
|---|---|
| **Cited articles** | This article cites 44 articles, 13 of which you can access for free at:<br>http://cancerres.aacrjournals.org/content/68/14/5997.full.html#ref-list-1 |

| | |
|---|---|
| **E-mail alerts** | Sign up to receive free email-alerts related to this article or journal. |
| **Reprints and Subscriptions** | To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org. |
| **Permissions** | To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org. |