A Systematic Approach to Analysing Gene-Gene Interactions: Polymorphisms at the Microsomal Epoxide Hydrolase EPHX and Glutathione S-transferase GSTM1, GSTT1, and GSTP1 Loci and Breast Cancer Risk

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Abstract

Objective: We undertook a case-control study in an Australian Caucasian population-based sample of 1,246 cases and 664 controls to assess the roles of detoxification gene polymorphisms EPHX T>C Tyr113His, GSTT1 deletion, GSTM1 deletion, and GSTP1 A>G Ile105Val on risk of breast cancer. Methods: We systematically addressed the main effects and possible gene-gene interactions using unconditional logistic regression to estimate odds ratios (OR) adjusted for potential confounders and using standard model building approaches based on likelihood theory. Results: There was a decreased risk associated with the EPHX CC genotype [OR, 0.60; 95% confidence interval (95% CI), 0.43-0.84; P = 0.003], marginally significant evidence of increased risk with GSTM1 null genotype [OR, 1.21; 95% CI, 1.00-1.47; P = 0.05], but no association with GSTT1 null genotype [OR, 1.12; 95% CI, 0.86-1.45; P = 0.4] or GSTP1 [OR, 0.95; 95% CI, 0.82-1.10; P = 0.5] genotype. The full model with all interactions gave a significantly better fit than a main-effects-only model (P < 0.001), providing evidence for gene-gene interactions. The most parsimonious model included main effects for EPHX, GSTT1, and GSTM1; a two-way interaction between EPHX and GSTM1; and a three-way interaction between EPHX, GSTM1, and GSTT1. Predicted risks were greatest for women carrying deletions of both GSTT1 and GSTM1, with either the EPHX TC genotype (OR, 2.02; 95% CI, 1.19-3.45; P = 0.009) or EPHX CC genotype (OR, 3.54; 95% CI, 1.29-9.72; P = 0.14). Conclusion: Detoxification gene polymorphisms may interact with each other to result in small groups of individuals at modestly increased risk. We caution against overinterpretation and suggest that pooling of similarly large studies is needed to clarify the possible role of such complex gene-gene interactions on breast cancer risk. (Cancer Epidemiol Biomarkers Prev 2007;16(4):769–74)

Introduction

Genetic risk factors for breast cancer include dominantly inherited deleterious mutations in the BRCA1 and BRCA2 genes. However, carriers of such mutations are rare at the population level and account for little of the familial aggregation observed for breast cancer (1). Thus, it is possible that more commonly occurring “low-risk” genetic factors explain a substantial proportion of the residual familial aggregation (2). It has been argued that these effects may be evident only when considering the joint effect of several genetic and environmental factors that act within a biological pathway (3), although searching for these interactions requires some care to reduce the frequency of making false-positive conclusions (4).

Candidate breast cancer predisposition genes include those that are involved in the metabolism of carcinogens that contain allelic variants that affect gene expression or protein function. These include the phase I microsomal epoxide hydrolase gene EPHX and the phase II glutathione S-transferase genes GSTT1, GSTM1, and GSTP1. EPHX encodes the epoxide hydrolase protein mEH, which catalyses the hydrolysis of arene and aliphatic epoxides to trans-dihydriodols (5), and typically results in detoxification and preparation for phase II conjugation reactions. The GST genes catalyze the glutathione-mediated reduction of exogenous and endogenous electrophiles with broad and overlapping substrate specificity (6, 7), generally producing readily excretable water-soluble compounds. Thus, allelic variants associated with altered (faster or slower) detoxification rates of potential carcinogens may confer an increased susceptibility to cancer, perhaps more so in the presence of environmental stresses such as smoking and UV light exposure (8).

A number of candidate functional variants exist for the EPHX and GST genes. The EPHX exon 3 T to C Tyr113His amino acid substitution variant was shown to have functional significance in vitro (9), with the His113 variant demonstrating 60% activity relative to the wild type Tyr113. Deletion variant or
null alleles exist for the GSTT1 and GSTM1 genes (10-12) and present biochemically as a failure to express protein. These alleles are common, and GSTT1 and GSTM1 null genotypes occur in ~10% to 20% and 50% of the Caucasian population, respectively (13). The GSTP1 exon 5 A to G Ile105Val polymorphism is one of several variants reported for this locus (14, 15), and the Val-containing isoform was shown to have altered specific activity and decreased heat stability (16-18).

These and other polymorphisms in the above-mentioned genes have been investigated as risk factors for numerous cancers, either independently or in combination. Results from individual case-control studies assessing the effect of GST polymorphisms on risk of breast cancer have been conflicting. Two meta-analysis studies (see Discussion) found a significant association of breast cancer with the GSTM1 deletion genotype for postmenopausal women (19, 20), with stronger evidence and higher odds ratios (OR) from studies in populations with a low frequency of the GSTM1 null genotype (20). One study reported a slight, marginally significant increase in risk associated with the GSTT1 deletion genotype (19). However, a recent pooled analysis (see Discussion) by Vogel et al. (21) found no significant association with breast cancer risk overall for GSTT1 or GSTM1, for either premenopausal or postmenopausal women. Meta-analyses of the GSTP1 exon 5 A>G Ile105Val polymorphism found no evidence that this variant was associated with risk for either the heterozygous genotype (21) or the homozygous genotype (19, 21). Furthermore, in the pooled analysis (21), there was no evidence that the GSTT1, GSTM1, or GSTP1 genotypes acted in combination to increase breast cancer risk, or interacted with smoking or reproductive history to modify breast cancer risk.

The only published study investigating the effects of EPHX on breast cancer (22) reported a not significant 1.5-fold increased risk associated with the EPHX exon 3 CC genotype compared with the wild-type TT genotype and a borderline significant 2.2-fold increased risk for EPHX exon 3 CC genotype in combination with GSTM1 null genotype. However, this hospital-based study of mostly postmenopausal women was relatively small—consisting of 238 cases and 313 controls.

In view of the uncertainty in the literature, we have undertaken a case-control study in Australia to assess the role of GSTT1, GSTM1, GSTP1, and EPHX genetic polymorphisms as risk factors for breast cancer. We specifically undertook a systematic analysis of the possible interactions between these genetic polymorphisms.

Materials and Methods

Subjects. The Australian Breast Cancer Family Study, a population-based, case-control-family study of breast cancer in women less than 60 years old, was carried out in Melbourne and Sydney with an emphasis on early-onset disease (23). Details of data collection methods have been described previously (23, 24). Briefly, cases were women diagnosed with a first primary breast cancer, identified through the Victoria and New South Wales cancer registries, and controls were women with no previous breast cancer selected from the electoral rolls (adult registration for voting is compulsory in Australia) by a stratified random sampling, frequency-matched for age. Questionnaires were used to measure risk factors; family history of cancers was systematically collected for each case and control from multiple sources in the family; and blood samples were collected at the time of interview from subjects agreeing to participate in genetic studies. Genotyping was done on the basis of DNA availability. Approval for the study was obtained from the ethics committees of The University of Melbourne, The Cancer Councils of Victoria and New South Wales, and the Queensland Institute of Medical Research. All subjects provided written informed consent.

Individuals included in genetic analyses (86% of participating subjects) differed from the remainder of participating subjects with respect to certain factors shown previously to be associated with breast cancer (25), namely family history (defined as having at least one first- or second-degree relative with breast cancer), oral contraceptive use, and parity. Ethnicity was self-reported. Subjects with any Australian aboriginal, Torres Strait Islander, or Maori heritage; or country of birth in the South Pacific, Indian Ocean, Caribbean islands, or Asia were classified as non-Caucasian. Preliminary analyses indicated that genotype distributions for GSTT1, GSTP1, and EPHX differed between individuals reporting Caucasian and non-Caucasian ethnicity, consistent with other studies (see Discussion). Further analyses were thus restricted to participants who identified themselves as Caucasian. In addition, molecular analyses to date (24) have identified 59 Caucasian cases carrying a deleterious germ line mutation in BRCA1 or BRCA2, and these participants were also excluded from the analyses. Final analyses included 1,246 cases and 664 controls.

Genotype Measurement. Genotyping was as described previously (26, 27). Briefly, PCR-agarose methodology was used to detect the GSTTT1 and GSTM1 homozygous deletion genotypes, and the ABI Prism 7700 Taqman Sequence Detection System methodology was used for genotyping the EPHX T to C Tyr113His polymorphism (rs1051740), and the GSTP1 A to G Ile105Val polymorphism (rs1695). In addition, because preliminary analysis indicated deviation from Hardy-Weinberg equilibrium, all individuals with EPHX CC and a subset of individuals with TC genotype were regenotyped using standard denaturing high-performance liquid chromatography and sequencing methodology. Primer sequences were as follows: forward, GCCCTCACATGGCTTC, and reverse, TTGGTTCTCAGAATCTCCTCAA, and PCR was done using a touchdown program with final annealing temperature of 55°C, and the denaturing high-performance liquid chromatography melting temperature of 59°C. This methodology was used to confirm genotype at the Tyr113His position and also to establish that no other variation, such as the reported Tmm9Stop variant, might be compromising assay results. Sequence confirmation of all aberrant denaturing high-performance liquid chromatography profiles detected during this rescoring revealed no evidence of additional variation under Taqman primer and probe binding sites, and identified genotype misclassification in 0.5% of cases and 0.2% of controls, with overcalling of the CC genotype for the Taqman genotyping.

Statistical Analysis. Hardy-Weinberg equilibrium was tested by Pearson's χ² test. Association of case-control status with genotype and environmental factors was modeled using unconditional logistic regression. EPHX and GSTP1 were initially modeled as codominant genotypes. Subsequently, EPHX and GSTP1 were included as linear terms coded 0, 1, or 2, with the homozygote wild-type (TT for EPHX, AA for GSTP1) coded as 0. The likelihood ratio test was used to assess any improvement of fit between the codominant genotype model and linear model for EPHX and GSTP1. GSTM1 and GSTT1 were each coded so that the homozygote deletion was contrasted with the heterozygote and wild-type pooled, reflecting the sensitivity of the gel-based genotyping methods used.

In addition to the genotype, a number of environmental factors considered to be potential confounders were also included: reference age categories (<30, 31-40, 41-50, >50

Unpublished data.
years), menopausal status, age at menarche quartile, number of live births (0, 1, 2, 3, ≥4), family history (any first- or second-degree relative reported to have had breast cancer), previous benign breast disease, body mass index [weight (kg)/height (m)^2] quartile, smoking (never/ever), ever drinking alcohol regularly, oral contraceptive use (never/ever), hormone replacement therapy ever, education (three categories), country of birth, and current Australian state of residence.

A systematic model building procedure was used to include or exclude the genetic polymorphisms and their interactions, using the likelihood ratio test to calculate the statistical significance of nested models (28). Both forward selection and backward elimination were used. In addition to the main effects of EPHX, GSTT1, GSTM1, and GSTP1, we considered all possible two-, three-, and four-way gene-gene interactions. Each genetic model was considered without including any environmental factors, including all those listed above, and including only those that remained significantly associated according to the Wald test. Due to the role of these candidate genes in detoxification, we also considered the interaction of each allele with smoking and regular drinking.

Results were presented as a lattice showing, for each model, the G statistic defined as twice the difference in log likelihoods between that from the fitted model and that from the null model (hence, G for the null model = 0). The first level shows the null model, the next level shows models with 1 degree of freedom (df), the next with 2 df, and so on. Each level considers all possible combinations of variables. Because including an effect for GSTP1 made no significant difference to any of the fitted models (see Results below), we have omitted it from the lattice to simplify the presentation (see Fig. 1). Differences in G statistics between nested models are compared with the χ^2 distribution with df = difference in the df of the nested models.

Whenever evidence of a gene-gene interaction with cancer risk was found, we tested for nonindependent assortment separately in cases and controls using a log-linear model (29). The same approach was used to test for associations between genotype and smoking, and between genotype and regular drinking. Finally, the analyses were repeated with the environmental factors omitted. After estimating the final models, the correlation matrix of the estimates was checked for evidence of collinearity. A significance level of 5% was chosen a priori and all tests were two-sided. Stata software was used for the analysis (version 9.0, StataCorp).

Results

The genotype distributions for GSTT1, GSTP1, and EPHX differed between individuals reporting Caucasian and non-Caucasian ethnicity (P < 0.001; P = 0.013; P = 0.002,
Table 1. Crude and adjusted univariate ORs for association between breast cancer and EPHX, GSTP1, GSTM1, and GSTT1 genotypes

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Controls (%)</th>
<th>Cases (%)</th>
<th>Adjusted* OR (95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>EPHX TT</td>
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<tr>
<td>TC</td>
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<td>CC</td>
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<tr>
<td>GSTP1</td>
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<td>AA</td>
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<td>GSTM1</td>
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<td>Present</td>
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<tr>
<td>Null</td>
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<td>GSTT1</td>
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<td></td>
<td></td>
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<tr>
<td>Present</td>
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<tr>
<td>Null</td>
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</tbody>
</table>

*Adjusted for age, education, family history (any reported first degree or second degree), previous benign breast disease, state, country of birth, hormone replacement therapy ever, and menopausal status. Initially also adjusted for body mass index, age at menopause, and malignant status. No difference between the crude ORs and adjusted ORs.

respectively). Therefore, further analyses were restricted to women who identified themselves as Caucasian. This included 663 controls and 1,238 cases genotyped for EPHX; 649 controls and 1,232 cases genotyped for GSTP1; 664 controls and 1,246 cases genotyped for GSTT1 and GSTM1, respectively (Table 1).

Evidence of deviation from Hardy-Weinberg equilibrium in controls (fewer heterozygotes than expected) was observed for EPHX and persisted after confirmation of genotypes using alternative genotyping methodology (P < 0.01). There was no evidence of deviation from Hardy-Weinberg equilibrium in controls for GSTP1 (P = 0.6).

In no instance were substantial differences found between the unadjusted and adjusted estimates. Adjusted estimates are reported in the text. As shown in Table 1, a significant decrease in breast cancer risk was associated with the EPHX CC genotype when compared with the TT genotype [OR, 0.60; 95% confidence interval (95% CI), 0.43-0.84; P = 0.003; trend OR, 0.83; 95% CI, 0.72-0.96; P = 0.01]. There was no association of breast cancer risk with GSTP1 genotype (GG versus AA OR, 0.90; 95% CI, 0.66-1.25; trend OR, 0.95; 95% CI, 0.82-1.10). The codominant model for EPHX and GSTP1 did not provide a significantly better fit than the linear model (P = 0.1 and P = 0.9, respectively). There was marginally significant evidence of increased breast cancer risk with GSTM1 null genotype (OR, 1.21; 95% CI, 1.00-1.47; P = 0.05), but not with GSTT1 null genotype (OR, 1.12; 95% CI, 0.86-1.45; P = 0.4).

A marginally significant interaction was found between drinking (never/ever) and GSTP1 (interaction OR, 1.36; 95% CI, 1.01-1.83; P = 0.04) and a suggestive association between smoking (never/ever) and GSTP1 (interaction OR, 1.30; 95% CI, 0.97-1.73; P = 0.09). Because a weak interaction could be the result of a population-level association between behaviors and genotype, these associations were tested for the controls only and found to be not significant for smoking (OR, 0.88; 95% CI, 0.65-1.21; P = 0.4) and drinking (OR, 0.83; 95% CI, 0.61-1.15; P = 0.3). Interactions between genotype and menopausal status and other reproductive factors such as parity and age at menarche were also not significant (data not shown).

The results of both forward selection and backward elimination excluded all main effects and gene-gene interactions with GSTP1 (P from 0.3 to 0.95). Therefore, for simplicity, the raw data for cases and controls in Table 2, and results from the model building procedure illustrated by Fig. 1, was shown excluding GSTP1.

The main-effects-only model gave a significantly better fit than the null model (χ² = 9.94 on 3 df; P < 0.02), even after adding the nonsignificant effect for GSTP1 (P < 0.05; data not shown). The full model also gave a significantly improved fit compared with the null model (χ² = 31.15 on 7 df; P < 0.0001), even after adding eight nonsignificant effects for all interactions with GSTP1 (P < 0.01; data not shown). The full model also gave a significantly better fit than the main-effects-only model (χ² = 21.21 on 4 df; P < 0.001), even after allowing for GSTP1 (P < 0.02). Therefore, there was evidence for the presence of gene-gene interactions.

The most parsimonious fit was provided by the highlighted model in Fig. 1 with 5 df, with G = 30.26. The fit was significantly better than the best models with 4 df (P = 0.02), 2 df (P = 0.003), or 1 df (P = 0.0001). The best 3 df model was not nested within the most parsimonious model and cannot be formally compared, but an AIC difference of 11.2 suggests the final model gave a superior fit. More complex models did not provide better fits: the best 6 df gave G₆ = 30.55 (P = 0.6), whereas the 7 df model with all possible interactions gave G₇ = 31.15 (P = 0.6).

As described in Table 3, the most parsimonious model that arose from this procedure consisted of main effects for EPHX (OR, 0.92; P = 0.5), GSTT1 (OR, 0.85; P = 0.3), and GSTM1 (OR, 1.36; P = 0.02), a two-way interaction between EPHX and GSTM1 (interaction OR, 0.72; P = 0.03), and a three-way interaction between EPHX, GSTM1 and GSTT1 (interaction OR, 2.63 per EPHX C allele, P = 0.001). This implies an OR of 2.02 (95% CI 1.19-3.45; P = 0.009) for those without a valid copy of GSTM1 or GSTT1, and the TC genotype for EPHX. When combined with the EPHX CC genotype, this OR becomes 3.54 (95% CI, 1.29-9.72; P = 0.01). When GSTP1 was added back to the final model as a main effect, there was neither evidence of association as a main effect (OR, 0.95; P = 0.5) nor modification of the effects of the other genes (results not shown).

Table 2. Number of cases and controls by GSTM1, GSTT1, and EPHX genotype status

<table>
<thead>
<tr>
<th>Genotype</th>
<th>GSTM1 present</th>
<th>GSTT1 present</th>
<th>GSTM1 null</th>
<th>GSTT1 null</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Controls Cases</td>
<td>Controls Cases</td>
<td>Controls Cases</td>
<td>Controls Cases</td>
</tr>
<tr>
<td>EPHX TT</td>
<td>123</td>
<td>239</td>
<td>37</td>
<td>44</td>
</tr>
<tr>
<td>GC</td>
<td>108</td>
<td>185</td>
<td>22</td>
<td>35</td>
</tr>
<tr>
<td>CC</td>
<td>36</td>
<td>56</td>
<td>4</td>
<td>4</td>
</tr>
</tbody>
</table>
When the most parsimonious model was fitted to cases and controls separately by Poisson regression, the two-way interaction was significant only for the cases (\( P = 0.035 \)). There was no evidence of nonindependent assortment of EPHX and GSTM1 for the controls (\( P = 0.2 \)). However, there seemed to be a three-way association between EPHX, GSTT1, and GSTM1 for the controls. Although this dependence was also seen in the cases, the association was greater for the controls (estimated coefficient = 0.2; \( P = 0.002 \)) than for the cases (estimated coefficient = 0.2; \( P = 0.05 \)).

**Discussion**

Detoxication genes have long been considered plausible candidates genes for predisposition to breast and other cancers. Our case-control study of a sample of relatively early-onset breast cancer found evidence for a protective effect associated with the EPHX CC genotype, suggestive evidence for increased risk associated with the GSTM1 deletion genotype, and no association with the GSTT1 deletion genotype or GSTP1 variant genotypes overall. These associations were not modified by menopausal status or other reproductive risk factors, and there was no strong evidence for interaction with cigarette smoking or regular alcohol drinking exposure.

As these genes act in a common pathway, we systematically tested a priori for gene-gene interactions using a hierarchical statistical model. We found evidence that the addition of gene-gene interactions gave a better fit than main effects only, as models that included interactions gave a better fit than main effects alone.

The involvement of GST genes in breast cancer etiology has been widely investigated by a large number of small studies with conflicting results, and joint analyses of published data sets have provided no firm conclusions regarding their role in breast cancer risk. Meta-analysis of at least 2,000 cases and 2,000 controls from 10 to 15 studies of predominantly Caucasian women by Egan et al. (19) found a borderline significant association with GSTM1 in postmenopausal women only (OR, 1.14), a border-line significant increasing risk for the GSTT1 deletion (OR, 1.11), and no association with GSTP1 GG genotype (OR, 1.04). The meta-analysis of GSTM1 data from more than 2,000 cases and 2,000 controls from 10 studies by Sull et al. (20) also found no overall association for the deletion genotype, but a significant increased risk for postmenopausal women only (pooled OR, 1.19), particularly for populations with a low frequency of the GSTM1 null allele. The smaller pooled analysis of Vogel et al. (21) of seven case-control studies (558-1,899 controls, 921-2,033 cases) found no association for GSTM1 (OR, 0.98), GSTT1 (OR, 1.11), or GSTP1 (OR for CC genotype, 0.93), and no interactions with smoking exposure, hormonal risk factors, or combinations of these genes. Last, a meta-analysis of seven case-control studies (2,815 cases, 3,170 controls) specifically evaluating the risk associated with cigarette smoking by GSTM1 genotype reported that smoking was associated with risk only when GSTM1 is deleted (30). Although the overall sample size included in these meta-analyses would have given sufficient power to detect small alterations in risk, these meta-analyses are likely to have been flawed by several factors. These include design issues (hospital-based versus population-based) and failure to adjust for ethnicity. In relation to ethnicity, differences between studies are traditionally thought to reflect confounding by ethnic genetic background or unmeasured exposures which differ between study populations. Alternatively, such differences may relate (at least in part) to the fact that the GSTM1 deletion genotype differs in frequency across ethnic groups (ref. 20; this study), and even within Caucasians (37.5-64.0%). This has the potential to confound the results due to issues associated with assay design—the gel-based assay design used almost exclusively to date for genotyping this deletion variant has precluded detection of heterozygote null carriers separately from homozygote wild-type. Consequently, the proportion of heterozygotes within this pool will vary with allele frequency, as will the extent to which any codominant effect effectively mask the risk associated with the homozygous deletion. It is interesting to note that from the meta-analysis of Sull et al. (20), the risk for breast cancer was significantly increased for populations with a lower GSTM1 null allele frequency (< 50.4%), but not for populations with GSTM1 null allele frequency ≥50.4%. However, most importantly, these previous studies did not systematically evaluate the role of within pathway interactions as we have.

This is the first large study investigating the associations between EPHX and GST gene variants and breast cancer risk. Our findings of associations with the GSTT1 and GSTP1 genotypes individually are in line with the meta-analysis studies, but not our findings of the GSTM1 deletion genotype alone. We also found evidence of statistical interaction between genotypes, with a three-way interaction between EPHX, GSTT1, and GSTM1 genotypes, with an estimate that was much larger than for any individual genotypes or other epidemiologic/demographic variables included in the same adjusted model. This suggests that the risk of breast cancer from such gene-gene interactions may be higher than for many of the known epidemiologic risk factors of breast cancer. Similar evidence for a contribution to breast cancer susceptibility by two-way interactions between polymorphisms of likely functional relevance, including those within the carcinogen metabolism, has recently been presented (31).

Although there is a strong biological rationale for interactions between polymorphisms in the same pathway, we recognize that we have tested multiple hypotheses. Great
caution should be exercised when assessing interactions, especially those based on small cell sizes, even though they have been derived from a moderately large study. The importance of very large sample sizes in providing sufficient statistical power to investigate with confidence possible effect modifications or interactions has been highlighted by studies of the hormone-metabolizing gene CYP17 (32). We thus present the data in a way that others can combine with their own so as to try to replicate or refute our findings.

This study further highlights the need for pooling of comparable data from large population-based case-control studies to have any hope of establishing definitive gene-gene interactions in breast cancer predisposition. These studies should use consistent or comparable exposure measures to be able to assess effect modification by common environmental carcinogen exposures, and to adjust for factors such as socioeconomic status as a surrogate for unmeasured exposure(s). The role of the Breast Cancer Family Registry (33), and carcinogen exposures, and to adjust for factors such as socioeconomic status as a surrogate for unmeasured exposure(s). The role of the Breast Cancer Family Registry (33), and the recently established Breast Cancer Association Consortium (34), will be integral to confirmation of speculative findings such as those reported here.

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References

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