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Polymorphisms in Drug Metabolism Genes, Smoking, and *p53* Mutations in Breast Cancer

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Abstract

Polymorphisms in phase I and phase II enzymes may enhance the occurrence of mutations at critical tumor suppressor genes, such as *p53*, and increase breast cancer risk by either increasing the activation or detoxification of carcinogens and/or endogenous estrogens. We analyzed polymorphisms in *CYP1B1, GSTM1, GSTT1*, and *GSTP1* and *p53* mutations in 323 breast tumor samples. Approximately 11% of patients exhibited mutations in *p53*. Women with mutations had a significantly younger age of diagnosis ($P = 0.01$) and a greater incidence of tumors classified as

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stage II or higher ($P = 0.002$). More women with mutations had a history of smoking (55%) compared to women without mutations (39%). Although none of the genotypes alone were associated with p53 mutations, positive smoking history was associated with *p53* mutations in women with the *GSTM1* null allele [OR = 3.54; 95% CI = 0.97–12.90 $P = 0.06$] compared to women with the wild-type genotype and smoking history [OR = 0.62, 95% CI = 0.19–2.07], although this association did not reach statistical significance. To test for gene–gene interactions, our exploratory analysis in the Caucasian cases suggested that individuals with the combined *GSTP1* 105 VV, *CYP1B1* 432 LV/VV, and *GSTM1* positive genotype were more likely to harbor mutations in *p53* [OR = 4.94; 95% CI = 1.11–22.06]. Our results suggest that gene–smoking and gene–gene interactions may impact the prevalence of *p53* mutations in breast tumors. Elucidating the etiology of breast cancer as a consequence of common genetic polymorphisms and the genotoxic effects of smoking will enable us to improve the design of prevention strategies, such as lifestyle modifications, in genetically susceptible subpopulations.

Keywords

breast cancer; *p53*; polymorphisms; drug metabolism

INTRODUCTION

Breast cancer is the second leading cause of cancer-related deaths among women in the United States [1]. Some of the known risk factors for breast cancer include previous biopsy, radiation therapy, family history, and reproductive history, but these factors may account for only half of the diagnosed breast cancer cases [2]. Although the etiological factors that determine breast cancer incidence have not been completely identified, it is clear that both environmental and genetic factors play a role in breast carcinogenesis.

In vitro and in vivo animal studies provided evidence that exposure to environmental contaminants and/or endogenous estrogen could potentially lead to the development of breast cancer [3-5]. Li et al. [6] demonstrated that aromatic DNA adducts were found more frequently in the normal adjacent tissues of breast cancer cases than in a cancer free control population, supporting the contribution of environmental contaminants to breast cancer risk. The contribution of smoking to breast cancer is an area of controversy, as there is a potential anti-estrogenic effect attributed to smoking [7]. However, a review of the literature regarding smoking and breast cancer would suggest that smoking is unlikely to be protective [8]. A recent cohort study supports the association of smoking with breast cancer [9], and the Carolina Breast Cancer Study found mutations in *p53* more frequently in current smokers than non-smokers [10].

Potential breast carcinogens are not limited to exogenous compounds; endogenous estrogen may also be a factor in breast carcinogenesis. Several known risk factors are related to overall lifetime exposure to estrogen, such as early menarche or late menopause, as well as obesity [11], which is linked to extra-ovarian estrogen production [12]. Estrogen can stimulate cellular proliferation [13] as well as induce DNA damage following metabolism to the catecholestrogens and the 3,4-hydroxycatecholestrogen quinone (CE-3,4-Q), which can form depurinating adducts as well as generate reactive oxygen species [14,15].

Both exogenous and endogenous chemicals require metabolic activation by the phase I cytochrome P450 (CYP450) enzymes in order to cause DNA damage. If the reactive metabolites produced during phase I metabolism are not detoxified by phase II enzymes, such as the glutathione S-transferases (GSTs), permanent genetic damage may occur. This

suggests that an individual's ability to metabolize exogenous and endogenous carcinogenic agents may influence their risk for breast cancer.

CYP1B1 is expressed in both normal and cancerous breast tissue [16] and is capable of activating a broad range of potentially carcinogenic substrates, including PAHs [17] and estrogen [18]. The 4-hydroxylation of estrogen by CYP1B1 appears to be the predominant hydroxylation pathway and results in the formation of a potentially carcinogenic catecholestrogen metabolite [19]. Single nucleotide polymorphisms (SNPs) in exon 2 (*A119S*) and in exon 3 (*L432V*) have been shown to alter the enzyme's metabolic capabilities [16,20]. It has been suggested that the 432 V allele increases the catalytic activity of CYP1B1 [20] as well as the ratio of the 4-OH to 2-OH catecholestrogen metabolites produced [21,22], although others have found either no effect of the SNP on enzyme activity or suggested that the 432 L allele actually increased catalytic activity [16].

Polymorphisms have also been identified in GSTs. Both *GSTM1* and *GSTT1* are deleted in individuals with the respective null alleles [23], which may prevent detoxification of activated substrates. PAHs, such as those found in cigarette smoke, are known substrates of GSTM1, therefore, an absence of the enzyme could be a disadvantage in the detoxification of these carcinogens [24]. The predominant GST in breast tissue [25], GSTP1, has two functional SNPs located at codons 105 and 114, which result in amino acid substitutions of I105V and A114V [23]. These SNPs influence enzyme activity depending on the substrate being metabolized [23]. Although GSTP1 is also believed to conjugate GSH with the catecholestrogen quinone [26], the impact of the SNPs on this metabolism has not been determined.

The association of these polymorphisms and breast cancer risk has been analyzed in multiple studies. Some studies have found associations between breast cancer risk and either individual genotypes alone or when interacting with other risk factors [27-31], with few analyzing the *GSTP1* A114V SNP [32]. However, other studies have not confirmed these associations [33-35]. These conflicting outcomes may be because of the heterogeneity of women in breast cancer populations, as breast cancer in each individual patient likely results from the interaction of different environmental and genetic factors. Few attempts have been made to compare the frequency of occurrence and types of mutations observed at critical oncogenic loci with the ability of breast tissue to metabolize chemical carcinogens. In this regard, studies in a Japanese lung cancer population have found associations with SNPs such as the I462V or *Msp*I SNPs of the *CYP1A1* gene and the incidence of *p53* mutations in the lung tumor tissue [36]. The *p53* gene is a critical tumor suppressor gene [37] that is mutated in 15–30% of breast cancer cases [38] and has been associated with a poorer prognosis and a shorter survival time [39]. In breast cancer patients, both Nedelcheva Kristensen et al. [40] and Gudmundsdottir et al. [41] suggested that mutations in the *p53* gene were associated with polymorphisms in the GST enzymes. We have thus hypothesized that patients exhibiting mutations in *p53* are more likely to be inherently susceptible to environmental and endogenously produced toxicants because of their genetically determined ability to metabolize carcinogens, which would result in elevated levels of activated carcinogenic metabolites.

We conducted a case-only study to test the hypothesis that women with polymorphisms in the phase I enzyme, *CYP1B1*, and phase II enzymes, *GSTM1, GSTT1*, and *GSTP1*, will be at increased risk for a mutation in *p53*. As damage at tumor suppressor loci may contribute to poor prognosis, these studies may allow for the early identification of the subset of patients at increased risk for alterations to key regulatory genes whose products contribute adversely to the patho-biology of breast cancer.

MATERIALS AND METHODS

Study Populations

The patient population used in this study is similar to the one described in a previous study [42]. Breast cancer cases were recruited chiefly at the Breast Care Center at the Wake Forest University Baptist Medical Center, with some samples also provided by the Moses H. Cone Memorial Hospital and the University of North Carolina-Chapel Hill Lineberger Comprehensive Cancer Center, from ongoing studies conducted from November 1998 to May 2004. The study subjects were at least 18 yr of age, able to speak English and comprehend informed consent, and did not have a previous history of cancer. Each of the study subjects was given a detailed description of the study protocol and signed informed consent as approved by each institution's Institutional Review Board. A 20 ml blood sample was collected from all study subjects along with a self-administered questionnaire containing demographic information, established breast cancer risk factors, medical history, and family history. For each case participant, paraffin embedded breast tumor or the lymph node, if available, was retrieved for *p53* mutation analysis.

Genotyping

DNA, which was used both for genotyping polymorphisms in the drug metabolic enzymes and as matched controls in the *p53* mutation analysis, was isolated from 200 μl of the blood sample donated by each participant using the QIAamp® DNA Blood Mini Kit (Qiagen, Valencia, CA), as described in the manufacturer's protocol. For the breast cancer cases that lacked a blood sample, DNA was extracted from the normal appearing tissue on the slides prepared from the paraffin embedded tumor tissue obtained for that patient. DNA was isolated using a digestion buffer consisting of 400 μg/ml proteinase K, 1% Tween-20, and TE buffer (10 mM Tris-HCl, 0.1 mM EDTA). For the samples obtained from UNC, matched DNA samples were obtained from lymphoblastoid cell lines that had been established from the same patients, as described previously [43].

Genotyping of *GSTM1* and *GSTT1* were determined using a modification of a previously described multiplex polymerase chain reaction (PCR) technique [44,45]. The PCR products were analyzed for the presence or absence of the 480 bp *GSTT1* and 231 bp *GSTM1* PCR products, with the 158 bp *GSTM4* product serving as the internal control. For samples requiring the use of paraffin embedded tissue (PET) and some blood samples, the *GSTM1* primers were used along with a previously described *GSTT1* and β-*globin* primer set [46,47] (Table 1), resulting in PCR products of *GSTM1*: 231 bp, *GSTT1*: 111 bp, and β-*globin*: 268 bp.

Genotyping of the *GSTP1* I105V SNP was determined using PCR followed by restriction fragment length polymorphism. The *GSTP1* I105V PCR product was digested in a 30 μl reaction containing 20 U of *Bsm*B1 (NE Biolabs, Beverly, MA) and 1× NE Buffer 3 (100 mM NaCl, 50 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM dithiothreitol). PCR products were analyzed for either the wild-type 166 bp fragment or variant 94 and 72 bp fragments. The *CYP1B1* A119S and L432V and *GSTP1* A114V genotypes were analyzed using a previously described PCR-SSCP technique [48] adopted for use with the GenePhor Electrophoresis System (conditions in Table 1). The PCR reaction mixtures were similar for each gene/exon: 2.5 mM $MgCl_2$, 0.2 mM (0.25 mM for the *GSTM1/T1* analyses from PET) dNTP mix (Promega, Madison, WI), 0.2 μM of each primer, 1–2 U of either Ampli*Taq* Polymerase Gold (Applied Biosystems, Foster City, CA) or *Taq* DNA polymerase (Eppendorf, Westbury, NY) with the manufacturer's supplied buffer mix, and 2 μl of template DNA.

Further into the study, genotyping for the *CYP1B1* and *GSTP1* SNPs was completed with the assistance of the Wake Forest University School of Medicine Center for Human Genomics using MALDI-TOF mass spectrometry MASSArray® (Sequenom®, San Diego, CA) as well as by sequencing with the assistance of either the Wake Forest University School of Medicine Biomolecular Resources Facility DNA Sequencing Core or MWG-Biotech, Inc. (High Point, NC).

For all of the assays utilized to determine SNPs in *CYP1B1* and the *GSTs*, initial experiments using either cell lines or tissues containing known SNPs were performed and the resulting gene products sequenced to verify that the assays could accurately determine the individual SNPs in each gene/exon.

p53 Mutation Analysis

Multiple 5 μm sections were cut from each of the PET samples. The investigator, along with the collaborating pathologist, extensively reviewed H&E stained slides prior to microdissection of the tissue sample. Each slide used in LCM was deparaffinized and stained using a modification of an H&E staining procedure defined by the National Institute of Environmental Health Sciences (NIEHS, dir.niehs.nih.gov/dirlep/lcm/protocols.html) for use in LCM. The deparaffinized slide was then microdissected using the PixCell II Microdissection Station (Arcturus, Mountain View, CA) with the CapSure™ Macro LCM caps (Arcturus). The cells were digested and released from the caps by incubating overnight at 37°C in an incubator with a digestion buffer consisting of 400 μg/ml proteinase K, 1% Tween-20, and TE buffer (10 mM Tris-HCl, 0.1 mM EDTA). Following overnight digestion, the samples were heated to 95°C for 8 min to deactivate the proteinase K. Multiple caps for each sample were collected if sufficient tissue was available.

Aliquots (5–10 μl) of the digest were utilized to amplify tumor DNA for two rounds of PCR for exons 5–9 of the *p53* gene using the nested primers listed in Table 2. The amplification reaction mixture consisted of 2.5 mM $MgCl_2$, 0.2 mM dNTP mix (Promega), 0.2 μM of each of the forward and reverse primers, 4 U of either Ampli*Taq* Polymerase Gold (Applied Biosystems) or *Taq* DNA polymerase (Eppendorf) with the manufacturer's supplied buffer mix, and 5–10 μl of digest. Following an initial denaturation step of either 2 (for Eppendorf *Taq*) or 10 (Ampli*Taq*) minutes, DNA was amplified by 40 cycles of denaturation for 30 s at 94°C followed by 2 min of annealing (see Table 2 for temperatures) and extension at 72°C. Primary PCR products were purified using the PerfectPrep® PCR Cleanup 96 Kit (Eppendorf) prior to secondary amplification. The cycles were reduced to 25 and annealing and extension times were shortened to 1 min for the secondary amplification reaction. Five to ten microliters of primary or purified primary PCR product was used as template for the secondary amplification. Amplification from the matched blood sample, which did not require a secondary amplification reaction, utilized the secondary primer set and 40 cycles of 30 s denaturation and 1 min annealing and extension.

The *p53* PCR products were initially screened for mutations using single stranded conformation polymorphism (SSCP) with the GenePhor flatbed electrophoresis system (Amersham Biosciences, Piscataway, NJ). The denatured products were separated by electrophoresis on the GenePhor system at the temperature predetermined for each exon and using the recommended voltage and wattage described in the manufacturer's protocol. Following electrophoresis, the gel was stained with the Plus One DNA Silver Staining Kit (Amersham Biosciences) according to the manufacturer's protocol. All tumors exhibiting a band shift were re-amplified and reanalyzed to confirm the shift and eliminate the possibility of a *Taq*-induced error. All tumors exhibiting a repeated band shift were sequenced by either the Wake Forest University School of Medicine Biomolecular Resources Facility DNA Sequencing Core or MWG-Biotech, Inc. by either direct sequencing of the purified

secondary PCR product or, as required, by isolating the PCR products from the SSCP gel, precipitating, and re-amplifying from the eluted bands. Samples that were difficult to interpret because of the presence of a heterozygous insertion or deletion were sequenced following cloning of the PCR products using the TOPO TA® Cloning Kit (Invitrogen, Carlsbad, CA). As more high-throughput technology became available, approximately one third of the PCR products were screened for mutations as a contract service by SpectruMedix L.L.C (State College, PA) by Reveal™ Temperature Gradient Capillary Electrophoresis (TGCE) and samples analyzed by SpectruMedix using SpectruMedix Analytical Software. As for the SNP assays, initial studies were conducted using either DNA obtained from cell lines with known *p53* mutations or patient samples to ensure the assays could identify mutations—in these instances, samples were sequenced to confirm the results.

Statistical Analysis

The comparisons of categorical demographics between those women with and without *p53* mutations were assessed using chi-square or Fisher exact tests. Wilcoxon rank-sum tests were used to assess differences in continuous characteristics. Logistic regression was used to estimate and assess the significance of the genotype effects after adjustment for patient characteristics, including age, age of menarche, smoking history, and body mass index (BMI). All two-way interactions between genotypes and effect modifiers were considered initially, and a backwards stepwise algorithm was used to remove nonsignificant interactions from the model ($P > 0.05$). Interactions with the highest P value were excluded first and the model was repeated as described. Analyses were completed using the Statistical Analysis System (SAS Institute, Cary, NC).

To test for high-order gene-gene interactions, the multivariate adaptive regression splines (MARS)-logit model was utilized. The MARS-logit model [49] is a hybrid model that combines MARS [50] and the traditional logistic model. The model is overfit followed by dropping the terms that contribute the least to the model. The maximum number of basis function is used to control how big the model is in the first step and the final size decided by the degrees of freedom (df) charged per basis function. In this study, a 10-fold cross-validation was used for MARS model selection. If the model selected by 10-fold cross-validation was too small, three df were charged per basis function. We tested for 1-, 2-, and 3-way interactions.

In the MARS-logit hybrid model, MARS is applied as a variable screening tool and the selected terms, and their extended terms, are plugged in a logistic regression model, which has been described in Cook et al. [49]. Cook used forward-backward and backward-forward automatic selection in logistic models; however, this study used backward first and then dropped the insignificant variables. The BIC (Bayesian information criterion) [51] was used as the criteria for final model selection as this discourages model overfitting by penalizing models for adding variables and it is more conservative than Akaike information criterion. SAS version 9.1 (SAS Institute) and commercial MARS software (Salford systems, San Diego, CA) were used in this study.

RESULTS

Of the 323 (301 Caucasian, 22 African American) women analyzed for *p53* mutations in this study, 34 (11%) exhibited mutations in exons 5–9 of *p53*, which is less than the expected 15–30% reported previously in breast cancer patient populations [38]. Differences in populations may contribute to the lower frequency observed in this study [52,53]. Two of the women had more than one mutation (#3260 and #3854) and one woman had a complex mutation that consisted of a single nucleotide mutation and insertion in tandem, making a total of 36 mutations analyzed (see Table 3). As some of the women exhibited identical

mutations, there are thirty different loci involved. Twenty-six of the 30 mutations were single nucleotide mutations, three were small deletions, and one was the complex mutation described above. Of the 26 single nucleotide mutations, 19 were missense mutations, four were nonsense mutations, and three were intronic/splice site mutations. Two former and two current smokers have deletions and complex mutations.

These mutations may impact protein function. The three deletions and the complex mutation would result in either a frameshift (2 samples), a three base pair deletion (1 sample) found between codons 253 and 254 (resulting in a loss of one amino acid), or as seen in the complex mutation (1 sample) the introduction of a stop codon followed by a frame shift. The four nonsense mutations would be expected to produce truncated proteins. Three of the women had mutations in potential intronic splice sites; the functional significance of these mutations is unknown.

Missense mutations were the predominant single nucleotide mutations found, consistent with the results reported in the International Agency for Research on Cancer (IARC) database [54]. The most common type of mutation found in this population is G:C → A:T, which is also consistent with the most frequent type of mutation in breast cancer as determined in the IARC database [54]. The missense mutations consisted of 19 different amino acid substitutions at 16 different codons. Two different mutations occurred at codon 237 and three different mutations were seen at codon 248. Eight of the women in this study had a mutation at codon 248, which is a known mutation hotspot in breast cancer [54]. Information contained in the IARC database indicates that some of these missense mutations have functional implications in terms of protein function [54].

When the characteristics of the 34 women with a mutation in *p53* were compared to women without a mutation, age of diagnosis and stage of disease were significantly different (P = 0.01 and 0.002, respectively, Table 4). The women with a mutation had a significantly younger age of diagnosis than those without a mutation [median age for wild-type = 59.9, median age for mutant = 50.0] and were more likely to have stage II-IV disease (66.7% compared to 39.0%). The association between smoking status (ever/never) and *p53* status was of borderline statistical significance (P = 0.08), as women with a *p53* mutation tended to have a history of smoking. However, *p53* mutation status did not differ by race, BMI, family history, current smoking status, age of menarche, parity, number of children, or age of first live birth.

The association between polymorphisms in four phase I/II enzymes and *p53* mutations was evaluated. An univariate analysis found no association between *p53* mutation status and *GSTM1* or *GSTT1* null genotype (Supplemental Table 1). The crude OR analysis showed a weak but not significant association between *p53* mutation and the GSTP1 105 VV genotype [OR = 1.47; 95%CI = 0.48–4.51]. The *GSTP1* 114 AV genotype appears to be somewhat protective compared to the AA genotype [OR = 0.24; 95% CI = 0.05–1.18]. None of the women in this study were homozygous for the *GSTP1* 114 VV allele, eliminating the possibility of analyzing the impact when both alleles are variant. *CYP1B1* A119S or L432V genotype did not have an effect on *p53* mutational status. The multivariable models revealed significant interactions between *GSTM1* and smoking history (P = 0.023) and between *GSTP1* I105V and age of menarche (P = 0.030).

As shown in Table 5, a slightly higher prevalence of *p53* mutations was observed in breast tumors from former (12.9%) or current smokers (15.4%) compared to that in never smokers (8.1%). In general, smoking was associated with a higher but not significant prevalence of *p53* mutations [OR = 2.14, 95%CI = 0.82–5.57 for former smokers and OR = 1.09, 95%CI = 0.35–3.24 for current smokers]. Current smoking status was associated with a higher

prevalence of transversion mutations in *p53* [OR = 2.34, 95% = 0.46–11.85]. Furthermore, smoking increased the odds of harboring a *p53* mutation in women with the GSTM1 null genotype [OR = 3.81; 95% CI = 1.03–14.12] compared to women with the *GSTM1* positive genotype [OR = 0.56; 95% CI = 0.17–1.80]. When these results were adjusted for stage, the odds were still increased, but the interaction was only marginally significant [OR = 3.54; 95% CI = 0.97–12.90, $P = 0.06$] (Table 6). The other polymorphisms were not analyzed by smoking status in detail as there were no significant interactions. The risk of having a *p53* mutation increased as the age of menarche increased in women with at least one *GSTP1* 105 I allele (II/IV) [OR = 1.67; 95% CI = 1.24–2.24, $P = 0.001$] while the risk decreased as the age of menarche increased in women who had the *GSTP1* 105 VV genotype [OR = 0.11; 95% CI = 0.01–1.06, $P = 0.056$].

Examining individual genotypes did not yield statistically significant associations with *p53* mutations. Figure 1 presents an exploratory analysis into the impact of gene–gene interactions on mutations using the MARS-logit technique. For this pilot study, we only used the data from Caucasians, as significant interactions disappeared when data from African-Americans were included. These results suggest that the combined genotypes of *GSTP1* 105 VV, *CYP1B1* 432 LV/VV, and *GSTM1* positive genotype were associated with mutations in *p53* [OR = 4.94; 95% CI = 1.11–22.06] compared with women with other genotype patterns. As the sample size is small, it would be necessary to confirm these interactions in a larger study, but it supports the importance of examining combinations of genotypes when determining the impact of polymorphisms on mutations.

DISCUSSION

Since nearly half of all breast cancer cases diagnosed are of unknown etiology, it is important to identify genetic risk factors involved in the etiology of sporadic breast cancer. Multiple case/control studies analyzing associations between individual SNPs of phase I and phase II enzymes and breast cancer risk have provided conflicting results [22-30]. Thus, we utilized mutations at the *p53* gene locus as a biomarker for patients susceptible to genetic damage who may be more likely to exhibit associations between polymorphisms in drug metabolic enzymes and breast cancer risk. For this study, exons 5–9 were analyzed for mutations, as this region contains the DNA binding domain that is most frequently mutated in human cancer [54,55].

Women that had a mutation in *p53* exhibited a younger age of diagnosis than women without a mutation. Age is a significant risk factor for breast cancer, as the risk has been shown to increase as a woman gets older [11]. Interestingly, a younger age at diagnosis has been associated with a poorer prognosis [56]. Thus, our results demonstrating that women with mutations in *p53* had a younger age of diagnosis suggest that genetic susceptibility to mutation at oncogenic loci may result in earlier tumor onset in this susceptible sub-population of women. In this regard, Sidoni et al. [57] showed that expression of *p53* was more frequent in younger populations, although an analysis of *p53* mutations in a Brazilian population of breast cancer cases did not confirm a difference in age between women with and without mutations [53]. These disparate results could be because of different environmental exposures or genetic factors between the two study populations. Women with mutations also exhibited a higher stage disease, which has been observed in other studies [10]. A recent study by Carey et al. [58] demonstrated a statistically significant difference in *p53* mutations in the more aggressive subtypes of breast cancer. An increased incidence of *p53* mutations in the younger women would be consistent with the more aggressive disease and poorer outcome in terms of survival and prognosis.

The results of this study suggest that smoking may contribute to a higher prevalence of *p53* mutations ($P = 0.08$), which is consistent with a study showing that PAH adducts were seen in breast cancer cases more frequently than controls [6]. As mentioned previously, the Carolina Breast Cancer Study determined that current smokers had a significantly higher mutation frequency in their breast tissue compared to never smokers ($P = 0.02$) [10]. This suggests that even though smoking alone may not be directly associated with breast cancer risk, it may be associated with the induction of mutations in tumor suppressor genes, which could lead to more aggressive forms of the disease. While we found no effect of the *GSTM1* null genotype on the *p53* mutational status [OR = 0.90; 95% CI = 0.44–1.84], our results demonstrated that the *GSTM1* null allele was associated with an increase of *p53* mutations in women who smoked, although significance was marginal when adjusted for stage. This may be because of the small number of samples with mutations. GSTM1 conjugates reactive electrophilic compounds produced during phase I metabolism, thus deletion of the gene would be expected to reduce the detoxification of carcinogenic metabolites found in cigarette smoke. Previous studies support the association of *GSTM1* null alleles with DNA damage as these alleles have been associated with PAH adducts in cancer cases compared to controls [59]. Consistent with this study, significant interactions have been noted with the *GSTM1* null alleles and smoking and their association with DNA adducts in the normal adjacent tissue of breast cancer cases [60]. Similar interactions have also been noted with alcohol consumption as well, supporting the importance of gene–environmental interactions and using genetic markers [61].

Our analysis of individual SNPs and risk for breast cancer based on genetic damage at the *p53* gene locus did not demonstrate significant associations for any of the drug metabolic polymorphisms analyzed. However, a previous study by Nedelcheva Kristensen et al. [40] demonstrated an association between the *GSTP1* 105 IV/VV genotypes and *p53* mutations ($P = 0.055$). Gudmundsdottir et al. [41] also noted a nonsignificant but interesting trend for the 105 VV genotype and having a mutation in *p53* ($P = 0.19$) and a statistically significant increase in the frequency of the *GSTT1* null allele in breast cancer patients with a mutation in *p53* ($P = 0.019$). The differences between these studies and the current one may be attributable to differences in the populations, as the patients were recruited in Norway [40] and Iceland [41] compared to our North Carolina population. Differences in lifestyle could result in differences in environmental exposures. Neither of the previous studies [40,41] reported demographic information or made adjustments for potential confounders and effect modifiers. Indeed, other studies have shown that the types of mutations found in *p53* tend to vary geographically [52], which suggests that differences in exposure to environmental agents could impact the influence of polymorphisms on the generation of mutations.

With limited sample size, our results suggest that individual genetic polymorphisms in drug metabolic enzymes may not influence the *p53* mutational status of breast cancer patients. However, we demonstrated marginally significant interactions between the *GSTM1* genotype and smoking that influence the prevalence of *p53* mutations. In addition, the combined genotypes of *GSTP1* 105 VV, *CYP1B1* 432 LV/VV, and *GSTM1* wild-type exhibited an almost five-fold increase in risk of having a mutation in *p53* in Caucasian women. The *CYP1B1* 432 Leu allele has been associated with a lower level of B[a]P induced DNA adducts supporting the effect of the polymorphism on carcinogen induced DNA damage [62]. The association of the *GSTM1* wild-type allele in the MARS-logit model with breast cancer risk seems contrary to the results obtained with the smoking data and ability of *GSTM1* to detoxify toxic metabolites. *GSTP1* is the most prevalent GST found in breast tissue [31] and it is possible that, because of the specificity of the GSTs for specific substrates, the presence of *GSTM1* may inhibit the ability of *GSTP1* to detoxify specific xenobiotics because of glutathione depletion. This was suggested in a previous case/control study which found an association between the homozygous wild-type alleles and

breast cancer [63]. Alternatively, GSTM1 is also capable of forming more toxic compounds from some substrates instead of detoxifying them [64]. The effect of smoking on gene–gene interactions was not analyzed as the number of patients were too few to stratify by smoking status. In the case of smokers, the high levels of exposure to toxic chemicals may overwhelm the normal detoxification pathways, and *GSTM1* would then play a more protective role. Further studies utilizing animal models and cell culture systems are required to determine the mechanisms of detoxification in smoking and non-smoking patients.

Our results suggest that future studies should focus on the combined effects of gene–gene and gene–environmental interactions on the induction of *p53* mutations, which will have a significant impact on our understanding of genetics and gene–exposure interactions in breast tumor progression. With the rapid development of high-throughput genotyping methods, we are in an excellent position to rapidly translate genetic susceptibility information into health behavior promotion, as genetically susceptible sub-populations are more motivated to participate in behavior intervention, such as smoking cessation.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

GST	glutathione *S*-transferase
SNP	single nucleotide polymorphism
PET	paraffin embedded tissue
SSCP	single stranded conformation polymorphism
BMI	body mass index
MARS	multivariate adaptive regression splines

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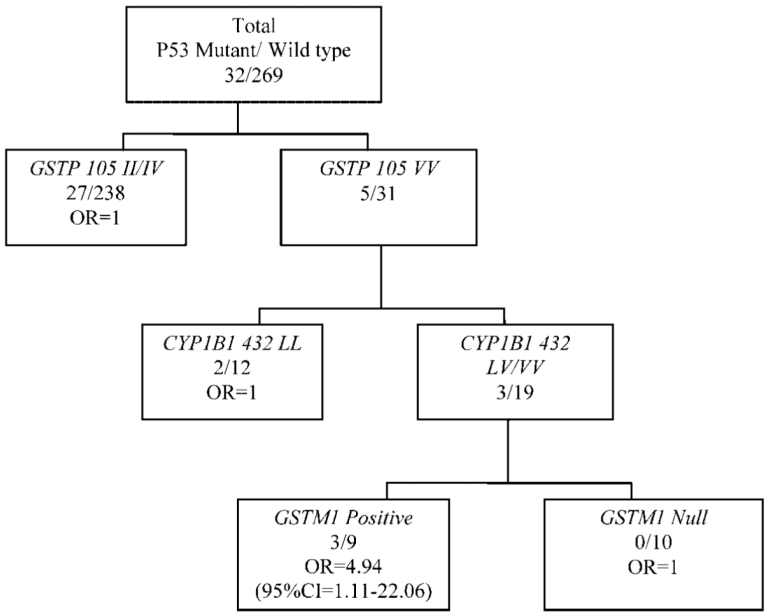

Figure 1.

Gene–gene interaction and *p53* mutations. Exploratory analysis of combined genotypes and *p53* mutations. The reported odds ratio was adjusted for age, age at menarche, BMI, and smoking status.

Table 1

Genotyping Primers, Annealing Temperatures, and SSCP Conditions

Primer	Oligo sequence	Annealing (°C)	SSCP Gel	SSCP Buffer	SSCP Temp.
GSTMP1	5′–CGC CAT CTT GTG CTA CATTGC CCG–3′				
GSTMP2	5′–ATC TTC TCC TCT TCT GTC TC–3′				
GSTMP3	5′–TTC TGG ATT GTA GCA GAT CA–3′	61	NA	NA	NA
GSTT1–5′	5′–TTC CTT ACT GGT CCT CAC ATC TC–3′				
GSTT1–3′	5′–TCA CCG GAT CAT GGC CAG CA–3′				
GSTT1(PET)–5′	5′–GCC CTG GCT AGT TGC TGA AG–3′				
GSTT1(PET)–3′	5′–GCA TCT GAT TTG GGG ACC ACA–3′	61.5	NA	NA	NA
Beta–globin–5′	5′–CAA CTT CAT CCA CGT TCA CC–3′				
Beta–globin–3′	5′–GAA GAG CCA AGG ACA GGT AC–3′				
GSTP1X5–5′	5′–AAC CCC AGG GCT CTA TGG G–3′	55	NA	NA	NA
GSTP1X5–3′	5′–GAA GCC CCT TTC TTT GTT–3′				
GSTP1X6–5′	5′–GAG AGT AGG ATG ATA CAT GG–3′	55	15/24	B	13°C
GSTP1X6–3′	5′–GGA ACA GCA TGG GGC CAG ATG–3′				
CYP1B1X2–5′	5′–TAC GGC GAC GTT TTC CAG AT–3′	61	SSCP	15/24	12°C
CYP1B1X2–3′	5′–CGT GAA GAA GTT GCG CAT CA–3′				
CYP1B1X3–5′	5′–ATG CGC TTC TCC AGC TTT GT–3′	60	15/24	15/24	10°C
CYP1B1X3–3′	5′–TCA GGT CCT TGT TGA TGA GG–3′				

Table 2

p53 Primers and SSCP Conditions

Exon	Primer	Oligo sequence	Size	Prim./sec. annealing (°C)	Gel	Buffer	SSCP temp.
5	3′ primary	5′–CCC TGT CGT CTC TCC AGC CC–3′		58			
	5′ primary/ secondary	5′–TTC CTC TTC CTA CAG TAC TC–3′[a]	212 bp	58	15/24	15/24	20°C
	3′ secondary	5′–CCC AGC TGC TCA CCA TCG–3′[a]					
6	3′ primary	5′–CTC CCA GAG ACC CCA GTT GC–3′		59			
	5′ primary/ secondary	5′–CCT CAC TGA TTG CTC TTA GG–3′[a]	147 bp	60	15/24	15/24	15°C
	3′ secondary	5′–AGT TGC AAA CCA GAC CTC AG–3′[a]					
7	5′ primary	5′–GGC GCA CTG GCC TCA TCT TG–3′		65			
	5′ secondary	5′–TGT GTT ATC TCC TAG GTT GG–3′[a]	143 bp	58	15/24	B	5°C
	3′ primary/ secondary	5′–TGG CAA GTG GCT CCT GAC–3′[a]					
	5′–sequencing	5′–TAT CTC CTA GGT TGG CTC TG–3′					
8	3′ primary	5′–CAC CCT TGG TCT CCT CCA CC–3′		58			
	5′ primary/ secondary	5′–TCC TAT CCT GAG TAG TGG T–3′[a]	166 bp	58	15/24	15/24	17°C
	3′ secondary	5′–TCC TGC TTG CTT ACC TCG–3′[a]					
9	5′ primary	5′–GGT GGA GGA GAC CAA GGG TG–3′		65			
	3′ primary	5′–AAC AGT CAA GAA GAA AAC GGC–3′	185 bp		15/24	15/24	12°C
	5′ secondary	5′–GCA GTT ATG CCT CAG ATT CAC–3′		58			
	3′ secondary	5′–GGC ATT TTG AGT GTT AGA CTG–3′					
	3′–sequencing	5′–TGA GTG TTA GAC TGG AAA CTT T–3′					

[a]indicates that the primer was previously described [65].

Table 3

Detailed *p53* Mutation Information

Lab ID	Smoking status	Codon	Base changes	Mutation type	Amino acid
		Missense mutations			
3647	NA	135	G → A	Transition	Cys → Tyr
3725	Former	143	T → A	Transversion	Val→ Glu
3109	Never	147	G → A	Transition	Val→ Ile
3260[a]	Never	151	C → T	Transition	Pro → Ser
3351	Never	156	G → C	Transversion	Arg → Pro
3050	Never	159	G → C	Transversion	Ala → Pro
3854[b]	Current	161	C → A	Transversion	Ala → Asp
3039	Former	172	G → A	Transition	Val→ Ile
5085	NA	193	C → T	Transition	His → Tyr
3663	Never	220	T → C	Transition	Tyr→ His
5137	Never	234	T → A	Transversion	Tyr→ Asn
3001	Current	237	T → A	Transversion	Met→ Lys
3682	Former	237	G → A	Transition	Met→ Ile
3030	Former	248	G → T	Transversion	Arg → Leu
3038	Former	248	C → T	Transition	Arg → Trp
3058	Never	248	C → T	Transition	Arg → Trp
3092	Never	248	C → T	Transition	Arg → Gln
3232	Never	248	G → A	Transition	Arg → Gln
3464	Never	248	G → A	Transition	Arg → Gln
3260[a]	Never	248	G → A	Transition	Arg → Gln
5262	Never	248	G → A	Transition	Arg → Trp
3975	Current	266	G → C	Transversion	Gly → Arg
3594	Current	273	G → A	Transition	Arg → His
5110	Former	273	G → A	Transition	Arg → Trp
3660	Never	282	C → T	Transition	Arg → His
		Nonsense mutations			
3070	Former	192	C → T	Transition	Gln → Stop

Lab ID	Smoking status	Codon	Base changes	Mutation type	Amino acid
3764	Former	196	C → T	Transition	Arg → Stop
5053	Never	204	G → T	Transversion	Glu → Stop
3151	Never	213	C → T	Transition	Arg → Stop
		Splice site mutations			
3091	Never	N14679 2 bp 5′	A → C	Transversion	NA
3854[b]	Current	N14680 1 bp 5′	G → A	Transition	NA
3645	Former	N14755 1 bp 3′	G → T	Transversion	NA
Lab ID	Smoking status	Codon	Coding sequence	Deleted sequence	Del./Ins. base
		Deletions and complex mutations			
5142	Former	229	GAC TGT ACC	GAC TAC C	GT
5126	Former	245	GGC GGC ATG	GGC GCA TG	G
3632	Current	253–254	ACC ATC ATC	ACC ATC	CAT
3610	Current	327–328	TAT TTC	TAA ATT C	A

[a,b]Indicates mutations from the same patient.

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Table 4

p53 Mutations by Personal Characteristics

Variable	Categories	Wild-type (N = 289)	Mutant (N = 34)	*P*-value
Race	Caucasian	269 (93%)	32 (94%)	1
	African-American	20 (7%)	2 (6%)	
Age	Mean (SD)	59.55 (12.51)	53.66 (13.16)	**0.01** *
	Median (Range)	59.90 (27.87–49.88)	49.95 (31.90–84.38)	
	Missing	0	0	
Family history	No (%)	209 (77%)	25 (78%)	0.9
	Mother/sister (%)	62 (23%)	7 (22%)	
	Missing	18	2	
Age menarche	≤12	136 (45.9%)	10 (13.3%)	0.11
	13–14	12 (36.4%)	16 (48.5%)	
	15+	39 (14.2%)	7 (21.2%)	
	Missing	17	1	
Smoking history	Never (%)	169 (61%)	15 (45%)	0.08
	Ever (%)	107 (39%)	18 (55%)	
	Missing	13	1	
Current smoker	No (%)	236 (88%)	26 (81%)	0.28
	Yes (%)	33 (12%)	6 (19%)	
	Missing	20	2	
BMI	Mean (SD)	27.61 (6.11)	25.71 (4.23)	0.18
	Median (Range)	26.26 (16.97–58.39)	25.36 (18.72–36.04)	
	Missing	18	3	
Number of children	Mean (SD)	2.15 (1.43)	2.03 (1.47)	0.56
	Median (Range)	2 (0–9)	2 (0–6)	
	Missing	12	1	
Parity	Nulliparous (%)	36 (13%)	5 (15%)	0.78
	≥1 child (%)	241 (87%)	28 (85%)	
	Missing	12	1	
Tumor stage[a]	0–I	175 (61.0%)	11 (33.3%)	**0.002** *
	II –IV	112 (39.0%)	22 (66.7%)	
	Missing	5	1	
Age at first live birth	Mean (SD)	23.38 (4.73)	25.18 (6.17)	0.17
	Median (Range)	23 (15.00–41.00)	25 (15.00–39.00)	
	Missing	15	1	

[a]According to the AJCC breast tumor staging guidelines.

*Statistically significant at P< 0.05.

Table 5

Association Between Smoking Status and *p53* Mutations

Smoking status	# mutations/# tumors	%	Crude OR (95% CI)	Adjusted OR[a] (95% CI)
Any p53 mutation				
Never	15/185	8.1	Reference	Reference
Former	11/85	12.9	1.69 (0.74–3.84)	2.14 (0.82–5.57)
Current	6/39	15.4	2.06 (0.75–5.70)	1.09 (0.35–3.24)
Transitions				
Never	10/185	5.4	Reference	Reference
Former	6/85	7.1	1.33 (0.47–3.79)	1.90 (0.59–6.13)
Current	2/39	5.1	0.95 (0.20–4.50)	0.40 (0.07–2.21)
Transversions				
Never	5/185	2.7	Reference	Reference
Former	3/85	3.5	1.32 (0.31–5.64)	1.01 (0.17–5.87)
Current	3/39	7.7	3.00 (0.69–13.12)	2.34 (0.46–11.85)

[a]Adjusted for age, age of menarche, BMI, and tumor stage.

Table 6

Interaction Between Smoking History and *GSTM1* Genotype in *p53* Mutations

GSTM1	Smoking status	*p53* wild-type n (%)	*p53* mutant n (%)	Adjusted OR (95%CI)[a]
Wild-type	Non-smoker	80 (59.3)	10 (58.8)	Reference
	Smoker	55 (40.7)	7 (41.2)	0.62 (0.19–2.07)
Null	Non-smoker	90 (62.5)	5(31.3)	Reference
	Smoker	54 (37.5)	11 (68.7)	3.54 (0.97–12.90)

[a]Adjusted for age, age of menarche, BMI, and tumor stage.