Genetic Polymorphism in Three Glutathione *S*-transferase Genes and Breast Cancer Risk

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ABSTRACT

The role of the glutathione *S*-transferase (GST) enzyme family is to detoxify environmental toxins and carcinogens and to protect organisms from their adverse effects, including cancer. The genes *GSTM1, GSTP1,* and *GSTT1* code for three GSTs involved in the detoxification of carcinogens, such as polycyclic aromatic hydrocarbons (PAHs) and benzene. In humans, GSTM1 is deleted in about 50% of the population, GSTT1 is absent in about 20%, whereas the GSTP1 gene has a single base polymorphism resulting in an enzyme with reduced activity. Epidemiological studies indicate that GST polymorphisms increase the level of carcinogen-induced DNA damage and several studies have found a correlation of polymorphisms in one of the GST genes and an increased risk for certain cancers.

We examined the role of polymorphisms in genes coding for these three GST enzymes in breast cancer. A breast tissue collection consisting of specimens of breast cancer patients and non-cancer controls was analyzed by polymerase chain reaction (PCR) for the presence or absence of the *GSTM1* and *GSTT1* genes and for *GSTP1* single base polymorphism by PCR/RFLP. We found that *GSTM1* and *GSTT1* deletions occurred more frequently in cases than in controls, and *GSTP1* polymorphism was more frequent in controls.

The effective detoxifier (putative low-risk) genotype (defined as presence of both *GSTM1* and *GSTT1* genes and *GSTP1* wild type) was less frequent in cases than controls (16% vs. 23%, respectively). The poor detoxifier (putative high-risk) genotype was more frequent in cases than controls. However, the sample size of this study was too small to provide conclusive results.

INTRODUCTION

The majority of chemical carcinogens like polycyclic aromatic hydrocarbons (PAHs) require metabolic activation before they can interact with cellular macromolecules and initiate cancer. The pathway by which ingested or inhaled environmental toxins are metabolized is the stepwise oxidative activation by phase I cytochrome P450 isozymes and detoxification through conjugation by phase II enzymes like glutathione *S*-transferases (GST). Conjugation of the reactive chemical species to glutathione renders xenobiotics water-soluble and facilitates their elimination from the body. Therefore, this reaction plays an important role in the inactivation of toxic and carcinogenic compounds. The phase II GST isozymes are encoded by at least four distantly related gene subfamilies, *GSTA, GSTM, GSTP*, and *GSTT* (Hayes and Pulford, 1995: nomenclature as suggested by Mannervik *et al.*, 1992). Each family may comprise several genes; for the M family at least five genes have been described, whereas only one gene is known for the P family and two for the T family.

The various GST isozymes have different, but often overlapping substrate specificities, and different patterns of expression in different cells and tissues (Awasthi *et al.*, 1993). *GSTA* and *GSTM* are highly expressed in liver. Most extrahepatic tissues express *GSTP1*. The *GSTP1* gene is involved in the development of acquired resistance towards anticancer drugs (Hayes and Pulford, 1995). *GSTT1* is expressed in human erythrocytes and in liver. GSTM1, which is also expressed in human lymphocytes, shows a high efficiency for conjugating epoxides and is particularly important for detoxification of PAH epoxides, the ultimate carcinogens of PAHs, but GSTP1 is also active towards PAH epoxides (Hayes and Pulford, 1995), and might compensate for loss of GSTM1 activity. GSTT1 catalyzes the conjugation of various smaller compounds, such as the industrial chemicals methyl chloride, methyl bromide, dichloromethane, ethylene oxide, and diepoxybutane, a reactive metabolite of 1,3-butadiene, in human liver and erythrocytes (Meyer *et al.*, 1991). This enzyme may have both detoxification and toxification activities depending on the substrate, it detoxifies the carcinogen ethylene oxide, but may enhance the formation of the genotoxic formaldehyde from dichloromethane.

GSTs are part of a complex integrated detoxification system with an intricate regulation of gene expression of phase I and phase II enzymes partially through the Ah receptor (Hayes and Pulford, 1995). Most GST-inducing compounds effect transcriptional activation through a response element (primarily the antioxidant-responsive element and the xenobiotic-responsive element). There seems to be an interdependance between regulation of various GST genes as well as expression of other detoxification systems. For example, a lack of the *GSTM1* gene results in decreased expression of another GST isozyme, *GSTM3* (Nakajima *et al.*, 1995) and reduces overall detoxification capabilities of affected individuals. In addition, the *GSTM1* null genotype seems to increase the inducibility of the cytochrome P4501A1 *(CYP1A1)* gene (Vaury *et al.*, 1995).

Interindividual differences in carcinogen metabolism, DNA repair, cell cycle control, programmed cell death, and other cellular functions all contribute to the carcinogenic process. Genetic polymorphisms are largely responsible for interindivdual traits; they can affect gene expression and protein function. The interindividual variations in carcinogen metabolism have been studied extensively and are recognized as an important

determinant of susceptibility to various cancers (Nebert *et al.*, 1996). In the metabolism of a procarcinogen, such as benzo[a]pyrene, a more efficient conversion of the procarcinogen to the ultimate carcinogen (the benzpyrene diol epoxide) by phase I enzymes can increase the risk of carcinogenesis, while a more rapid clearing by phase II enzymes can confer a protective effect. Variability in the level and activity of phase I and II enzymes is due, in part, to genetic polymorphisms. These can consist of single base substitutions or total gene deletions.

   The *GSTP1* gene has a single base change of an A to G that results in an amino acid change of Ile to Val, resulting in reduced enzyme activity (Harries et al., 1997, see Table 1). This variant has been associated with increased risk of cancer at various sites (Hirvonen, 1999). The GSTM1 gene is also polymorphic, containing two well-characterized, expressed genes and a null allele which reflects nearly complete deletion of the gene and results in total loss of GSTM1 activity (Seidegard *et al.*, 1988). This *GSTM1* gene deletion occurs quite frequently, in about 50% of the population, though there are large variations among different ethnic groups (Bell *et al.*, 1992). Individuals with the null allele are at an increased risk for various cancers including cancer of the lung and bladder (Bell *et al.*, 1993; McWilliams *et al.*, 1995). Studies in Japanese populations that consider interaction of *GSTM1* with smoking and/or CYP1A1 genotypes report a significant increase in lung cancer risk (Hayashi *et al.*, 1992, Nakachi *et al.*, 1992). *GSTT1* also exhibits a deletion polymorphism (Nelson *et al.*, 1995). Less is known about cancer susceptibility in individuals with *GSTT1* deletion, but several studies have consistently shown evidence of increased DNA damage in individuals with the null allele (Rebbeck, 1997). The combined *GSTM1* and *GSTT1* null genotypes have been associated with increased lung cancer risk (Kelsey *et al.*, 1997). The relative cancer risk associated with deletion of the *GSTM1* and/or *GSTT1* gene is probably small, but because of the high frequency of these deletion genotypes, there is the potential for broad population level impact. It has been estimated that the *GSTM1* null genotype accounts for 17% of lung cancer (McWilliams *et al.*, 1995), as well as 17% of bladder cancer cases (Brockmoeller *et al.*, 1994). This is in contrast to specific cancer genes such as p53 and BRCA1 which have a high individual cancer risk but low population frequency and a low population attributable risk, less than 10% of all breast cancers (Caporaso and Goldstein, 1995).

Table1: GST genetic polymorphisms investigated in this study

Gene	Base change	Functional effect	Detection method
GSTM1	Gene deletion	No enzyme	Allelele-specific PCR
GSTP1	A to G transition resulting in Ile105Val change	Reduced enzyme activity	PCR/RFLP as *Alw*261 site created
GSTT1	Gene deletion	No enzyme	Allelele-specific PCR

Because PAHs are known to cause mammary cancer in rodents, we hypothesize that they are involved in breast cancer development in humans. Variation in the metabolism of PAHs is likely to influence the risk of breast cancer. We have initiated studies analyzing a breast tissue collection of breast cancer cases and controls to evaluate how the efficiency of PAH detoxification, that is polymorphism in the *GSTM1, GSTP1,* and *GSTT1* genes, affects breast cancer susceptibility.

MATERIALS and METHODS

Breast tissue collection: A collection of breast tissue from breast cancer patients and cancer-free individuals established by Dr. Martha Stampfer, LBNL, was used. The tissue bank contains specimens from reduction mammoplasties and mastectomies including tumor, peripheral non-tumor tissue, and in a few cases contralateral tissue. The age and disease status of the specimen donors are known. Individuals undergoing reduction mammoplasty – the control group - ranged in age from 15 to 68 years, mastectomy patients' age ranged from 30 to 87 years. For polymorphism analysis of the case group either tumor or peripheral non-tumor tissue was used

Prevention of PCR contamination: We have taken several precautions to prevent contamination of reactions with PCR product to avoid false positive results. The steps involved in the PCR procedure are (1) sample preparation, (2) PCR reaction set-up, (3) amplification, product analysis and quantitation. Each step is performed at a different workstation located in adjacent rooms where the work area for step 2 cannot be accessed from the work area for step 3. Each workstation has dedicated supplies and equipment, which cannot be brought into another area. The PCR set-up area is in a dead-air box equipped with UV light. All PCR reactions include a negative control.

Table 2: Oligonucleotide primer sequences for genetic polymorphism detection

Gene	Oligonucleotides	Sequence	PCR product size (bp)
GSTM1	Upper primer	5'-CTGCCCTACTTGATTGATGGG-3'	273
	Lower primer	5'-CTGGATTGTAGCAGATCATGC-3'	
GSTT1	Upper primer	5'-TTCCTTACTGGTCCTCACATCTC-3'	480
	Lower primer	5'-TCACCGGATCATGGCCAGCA-3'	
GSTP1	Upper primer	5'-ACCCCAGGGCTCTATGGGAA-3'	176
	Lower primer	5'-TGAGGGCACAAGAAGCCCCT-3'	
β-actin	Upper primer	5'-GGGCACGAAGGCTCATCATTC-3'	320
	Lower primer	5'-TTTTTGGCTTGACTCAGGATT-3'	

Determination of GSTM1 and GSTT1 genotypes: The *GSTM1* and *GSTT1* genetic polymorphisms are determined simultaneously by multiplex PCR. For *GSTM1*, primers

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that hybridize to the 5' region of exon 4 and 3' region of exon 5 of *GSTM1* are used (Brockmöller *et al*., 1994; see Table 2). For *GSTT1*, the primers described by To-Figueras *et al*. (1997) are used. A β-actin fragment is amplified in parallel as a control for amplifiable DNA. PCR-reaction products are analyzed by gel electrophoresis (3% agarose) and ethidium bromide staining for the presence of a 273 bp *GSTM1* product, a 480 bp *GSTT1* product, and a 320 bp β-actin product. The lack of a *GSTM1* or *GSTT1* amplification product in the presence of an actin amplification product is consistent with the homozygous null genotype. In all reactions, a positive and a negative control are included. As a quality control, 10-20% of all samples are repeated as blinded duplicates.

Determination of GSTP1 genotype: The A to G polymorphism at codon 105 is determined by PCR of a 176 bp fragment and restriction fragment length polymorphism (RFLP): the product is digested with the diagnostic restriction enzyme *Alw*261, which does not cut the consensus sequence, but cleaves the *GSTP1* G allele to yield two restriction fragments of 91 bp and 85 bp length that can be analyzed by gel electrophoresis (Harries *et al*., 1997).

RESULTS

We determined *GSTM1, GSTP1,* and *GSTT1* polymorphisms in 101 breast tissue specimens from the tissue bank established by M. Stampfer (62 cases and 39 controls). The *GSTM1* and *GSTT1* status was determined by a multiplex PCR assay that detects the

Table 3: *GSTM1, GSTP1, GSTT1* genotypes detected in cases and controls

Gene	Allele	Cases		Controls		p-value **
		n	(%)	n	(%)	
GSTM1	*1	28	(45)	24	(62)	
	*0	34	(55)	15	(39)	0.11
GSTP1	Ile/Ile	30	(48)	17	(44)	
	Ile/Val	29	(47)	18	(46)	0.57
	Val/Val	3	(5)	4	(10)	
GSTT1	*1	51	(82)	34	(87)	
	*0	11	(18)	5	(13)	0.51

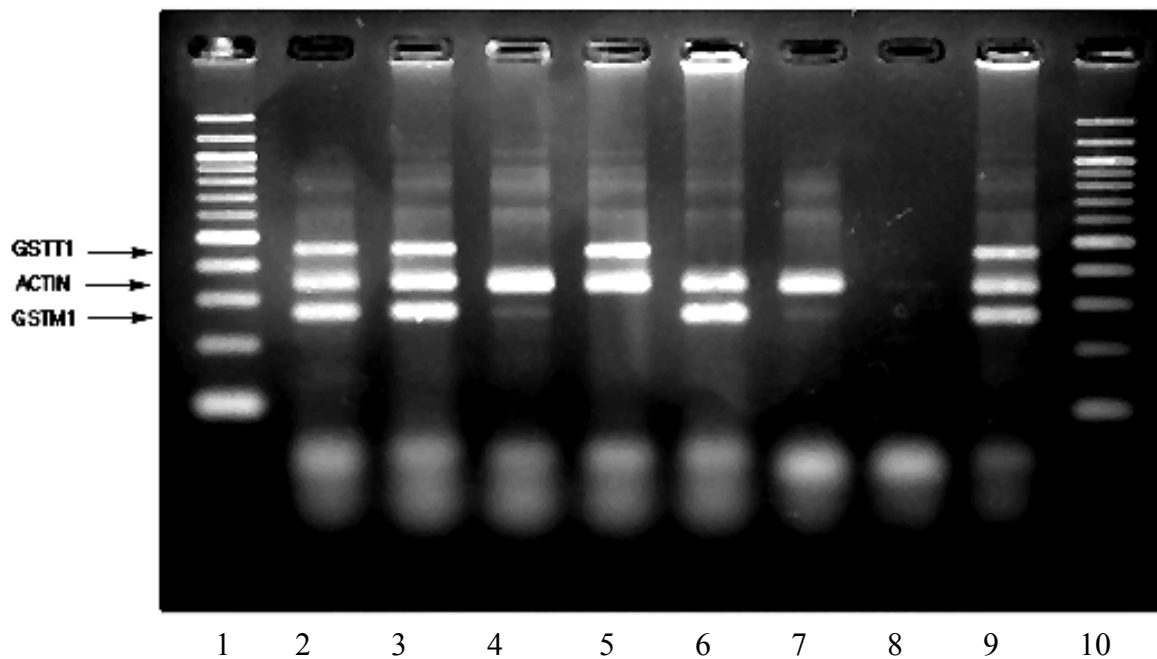*1, nondeleted genotype, *0, homozygous deletion
**Pearson Chi-Square test

Figure 1: Agarose gel of the PCR products for detection of GSTM1 and GSTT1 deletion polymorphisms in seven specimens. GSTM1 - 273 bp, GSTT1- 480 bp, and *-actin - 320 bp. Lane 1, 10, molecular weight standards; lane 2, 3, 9, GSTM1*1/GSTT1*1; lane 4, 7, GSTM1*0/GSTT1*0 ; lane 5, GSTM1*0/GSTT1*1; lane 6, GSTM1*1/ GSTT1*0; lane 8, negative control.

176 bp
91 bp
85 bp

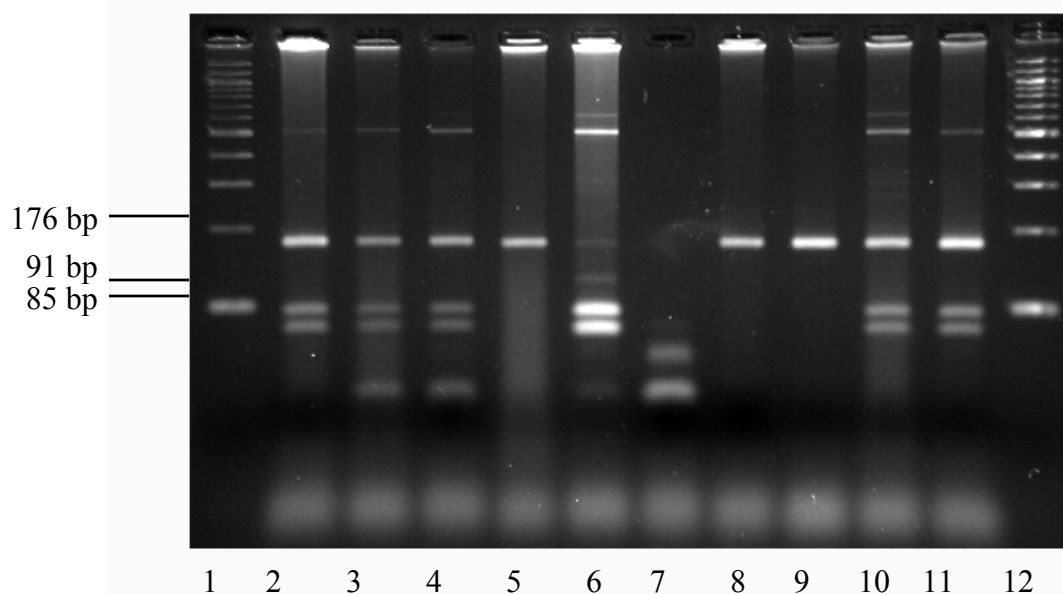1   2   3   4   5   6   7   8   9   10 11 12

Figure 2: Agarose gel of the GSTP1 PCR products after digestion with Alw261 for detection of GSTP1 polymorphisms. The 176 bp GSTP1 PCR product is digested with Alw261, which does not cut the wildtype A sequence, but cleaves the G sequence to yield two restriction fragments of 91 bp and 85 bp length. Lane 1, 12, molecular weight standards; lane 2, 3, 4, 10, 11, heterozygous variant allele (GSTP1 Ile/Val); lane 5, 8, 9, homozygous wild type for GSTP1 (GSTP1 Ile/Ile); lane 6, homozygous variant allele (GSTP1 Val/Val); lane 7, negative control.

presence of *GSTM1* and *GSTT1* fragments, either homozygous nondeleted or heterozygous (Figure 1). The *GSTP1* polymorphism was detected by PCR followed by restriction enzyme digestion that distinguishes single base polymorphism by RFLP (Figure 2). We found that the *GSTP1* polymorphism cannot be analyzed in the same multiplex PCR reaction as *GSTM1* and *GSTT1* because the *GSTM1* fragment is also digested by *Alu*26I to a fragment of similar size as the *GSTP1* fragment.

The results in Table 3 show a higher proportion of *GSTM1* and *GSTT1* homozygous deletions in the breast cancer group compared with the control group (55% vs. 39% for *GSTM1* deletion and 18% vs. 13% for *GSTT1* deletion), but a slightly higher proportion of the *GSTP1* (i.e., Ile/Val or Val/Val) variant genotype in control group than in the case group (56% vs. 52%).

The GST enzymes are involved in the detoxification of a variety of compounds and often have overlapping substrate specificity. We investigated whether certain profiles of GST genotypes may be associated with the risk of breast cancer. An effective detoxifier genotype with the presence of the *GSTM1* and *GSTT1* (*GSTM1*1, GSTT1*1*) gene and the homozygous wild type for *GSTP1* (*GSTP1* Ile/Ile) would be considered a low-risk genotype as Table 4 displays. A poor detoxifier genotype with GSTM1 and GSTT1 deletion (*GSTM1*0, GSTT1*0*) and a heterozygous or homozygous variant allele (*GSTP1* Ile/Val or *GSTP1* Val/Val) would be considered high-risk. The proportion of cases with an effective detoxifier genotype was less than that of controls (16% vs. 23%, respectively). There were only two individuals from the case and two from the control group with *GSTM1*0, GSTT1*0* and *GSTP1*Ile/Val. There were no individuals with *GSTM1*0, GSTT1*0* or *GSTM1*0* only and *GSTP1*Val/Val. Because GSTM1 and GSTP1 are responsible for conjugating PAH diolepoxides, which are considered the ultimate carcinogens of PAHs, the poor detoxifier genotype *GSTM1*0, GSTP1*Ile/Val

Table 4: Association between GST genotype profiles and breast cancer

Genotype	Allele	Cases		Controls	
		n	(%)	n	(%)
effective detoxifier	*GSTM1*1, GSTT1*1, GSTP1Ile/Ile*	10	(16)	9	(23)
poor detoxifier	*GSTM1*0, GSTP1Ile/Val*	16	(26)	8	(21)

The p-value for the comparison of the effective detoxifier genotype in cases and controls is p = 0.54, for the poor detoxifier genotype p = 0.38.

was considered. The proportion of cases with this genotype was more than that of controls (26% vs. 21%, respectively). Even though the data are suggestive of a trend of increased risk with poor detoxification genotype, there were no statistically significant differences between cases and controls.

DISCUSSION

Genetic variants of carcinogen-metabolizing genes show distinct ethnic variation. The frequency of the *GSTM1* deletion genotype ranges widely across different ethnic groups (from 30% among blacks to 88% among Samoans, Lin *et al.*, 1994). The *GSTT1* deletion polymorphism exhibits an even larger range in distribution between ethnic groups (Nelson *et al.*, 1995). The ethnic variation of *GSTP1* has not been studied in great detail. However, we have found that the *GSTP1* homozygous variant genotype is very high in a Hispanic population from the El Paso area, occurring with the same frequency as the wild type genotype (Sanchez et al., unpublished data) whereas, it occurred in less than 10% in English individuals (Harries et al., 1997).

Many low-penetrance genetic variants have been studied for their potential role in breast cancer (Dunning *et al*., 1999). Several reports in the literature examining whether *GSTM1* and *GSTT1* null genotypes represent susceptibility factors for breast cancer, found either no association (Bailey *et al*., 1998) or a very weak association (Ambrosone *et al*., 1995, Gudmundsdottir et al., 2001). Interestingly, the *GSTM1* and *GSTT1* deletion genotype increases survival after treatment for breast cancer either by reducing the detoxification of chemotherapeutic drugs or by preventing therapy-generated reactive oxidant damage (Ambrosone *et al*., 2001). A statistically significant difference in genotype frequency of PAH-metabolizing genes of cases and controls has been reported previously for the *GSTP1* Ile/Val polymorphism and for *GSTM1* deletion in postmenopausal women (Helzlsouer *et al*., 1998). Susceptibility genes as exemplified by carcinogen-metabolizing genes are strongly dependent on exposure to carcinogens. Studies in Japanese populations that considered the interaction between *GSTM1* and smoking found that lung cancer risk depended on the amount of cigarettes smoked (Nakachi *et al.*, 1992). A recent study found a correlation of *CYP1A1* genotype and breast cancer only among women with high PCB body burden (Moysich *et al*., 1999). Therefore, to assess the risk of a certain metabolic genotypes, one should take into consideration both the gene of interest and the exposure. Likewise, studies evaluating the risk of specific exposures should take into account interindividual genetic susceptibility. The studies mentioned above on *GSTM1* and *GSTT1* polymorphism and breast cancer found considered only exposure to tobacco smoke (Bailey *et al*., 1998, Ambrosone *et al*., 1995).

When considering low-penetrance traits like these GST polymorphisms one has to keep in mind that metabolic pathways like detoxification are very robust; systems are buffered because of the redundancy of enzymes. One GST gene can phenotypically compensate for genetic variation in another. Therefore, one should look at variants from multiple genes in a pathway. Only if several changes in the same pathway are present, is it likely that one will see an effect. We grouped subjects according to their genotype into an "effective metabolizer" group in which more effective detoxification is expected and a "poor metabolizer" group where, through deletion and single base substitution changes,

less effective detoxification is expected. Additionally, one should know the extent of the exposure in question, since enzymes are rarely saturated for their substrates. Only individuals with high exposure (to polycyclic aromatic hydrocarbons, PAHs, through smoking or occupational exposure, for example) are presumed to be at increased risk.

The present study reports our initial efforts to determine if interindividual variations in metabolic capacity influences the risk of breast cancer and, more specifically, if polymorphisms in genes involved in PAH metabolism are related to cancer susceptibility. Even though many epidemiological studies support a role of GSTs in cancer development, and our data show a similar trend, the observed effect was not statistically significant. These inconsistencies might be due to the limited number of samples available and, therefore, a lack of statistical power. Furthermore, we were unable to control for potential confounding factors, such as race, age at menarche, age at menopause, and age at first full-term pregnancy. The main purpose of this study was to establish the techniques of polymorphism analysis in our laboratory.

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