[CANCER RESEARCH 61, 7130–7135, October 1, 2001]

Polymorphisms in Glutathione *S*-Transferases (*GSTM1* and *GSTT1*) and Survival after Treatment for Breast Cancer[1]

Christine B. Ambrosone,[2] Carol Sweeney, Brian F. Coles, Patricia A. Thompson, Gail Y. McClure, Soheila Korourian, Manal Y. Fares, Angie Stone, Fred F. Kadlubar, and Laura F. Hutchins

Derald H. Ruttenberg Cancer Center, Mount Sinai School of Medicine, New York, New York 10029 [C. B. A.]; Division of Epidemiology, University of Minnesota, Minneapolis, Minnesota 55454 [C. S.]; National Center for Toxicological Research, Division of Molecular Epidemiology, Jefferson, Arkansas 72079 [B. F. C., G. Y. M., M. Y. F., A. S., F. F. K.]; Arkansas Cancer Research Center, University of Arkansas for Medical Sciences, Little Rock, Arkansas 72205 [S. K., L. F. H.]; and University of Texas M D Anderson Cancer Center, Department of Epidemiology, Houston, Texas 77030 [P. A. T.]

ABSTRACT

The response to treatment for breast cancer is likely predicted by a number of disease and tumor tissue characteristics, many of which are under active investigation. One area that has received little attention is that of endogenous capabilities to respond to reactive oxygen species and subsequent byproducts resulting from radiation therapy and a number of chemotherapeutic agents, preventing cytotoxicity toward tumor cells. The glutathione *S*-transferases are key conjugating enzymes in this response, and *GSTM1* and *GSTT1* have deletion polymorphisms that result in no enzyme activity. In this retrospective study, we evaluated the role of *GSTM1*- and *GSTT1*-null genotypes on disease-free and overall survival among 251 women who received treatment for incident, primary breast cancer. Women were identified through Tumor Registry records and normal archived tissue retrieved for genotyping. Adjusting for age, race, and stage at diagnosis, women with null genotypes for *GSTM1* and *GSTT1* had reduced hazard of death [adjusted hazard ratio (HR), 0.59; 95% confidence interval (CI), 0.36–0.97; and HR, 0.51; CI, 0.29–0.90, respectively] in relation to those with alleles present. Furthermore, women who were null for both *GSTM1* and *GSTT1* had one-third the hazard of death of those with alleles for both genes present (adjusted HR, 0.28; 95% CI, 0.11–0.70). Similar relationships were noted for risk of recurrence. These data indicate that interindividual differences in activity of enzymes that prevent therapy-generated reactive oxidant damage may have an important impact on disease recurrence and overall survival.

INTRODUCTION

Chemotherapy and/or radiation therapy after surgery for breast cancer significantly reduce the risk of recurrence and mortality. However, these treatments do not cure all patients, and considerable research has been focused on tumor tissue characteristics that may predict prognosis. Relatively little attention has been paid to possible underlying host factors that could play a substantial role in reduced treatment efficacy.

Both radiation therapy and chemotherapy largely exert their antineoplastic effects by generating ROS[3] and their byproducts (1, 2). In a population including patients receiving radiation therapy and those administered chemotherapy, marked increases in ROS levels were noted with spectrophotometric detection following treatment in both groups (3). Interestingly, no significant differences were observed between patients who received radiation or chemotherapy, indicating that both treatment regimens are equally effective in generating ROS. Because in many cases ROS are the proximate cause of tumor cell death, the amount of reactive species that reach tumor cells and have either direct cytotoxic effects or trigger intracellular apoptotic pathways is likely to have initial and immediate impact on treatment efficacy. Thus, interindividual variability in enzymes that will affect ROS levels is likely to impact patient prognosis after treatment.

The GSTs are induced under conditions of oxidative stress, and α-, π-, μ-, and θ-class GSTs are active in detoxification of numerous products resulting from reactive oxidant damage to DNA and lipids, such as organic epoxides, hydroperoxides, and unsaturated aldehydes (4). GST-catalyzed reduction of these molecules prevents further oxidant damage within cells. GSTs M1 and T1 have been shown to have activity toward lipid hydroperoxides (5),[4] and individuals lacking each of these enzymes may have reduced removal of secondary organic oxidation products produced by cancer therapy and, thus, may have better prognoses. In this retrospective analysis of women who were treated for breast cancer, we evaluated the role of genetic polymorphisms in *GSTM1* and *GSTT1* on disease-free and overall survival after treatment.

MATERIALS AND METHODS

Study Population. For this study, women (n = 251) were identified through the Arkansas Cancer Research Center Tumor Registry as described previously (6). The study was approved by the Institutional Review Board of the University of Arkansas for Medical Sciences. Eligibility criteria included a diagnosis of pathologically confirmed incident, primary, invasive breast cancer; first course treatment with radiation or chemotherapy; and availability of normal archived tissue for genotyping. Information regarding demographics, disease characteristics (stage, grade, and estrogen and progesterone receptor status), courses of treatment(s), and vital and recurrence status was obtained from the Tumor Registry and/or pathology reports. The Arkansas Tumor Registry actively conducts annual follow-up on all patients. Among 177 women alive at last contact, 143 had follow-up dates between September 1998 and August 1999 and 30 had follow-up dates between September 1997 and August 1998. Only four living patients had last contact dates earlier than September 1997. Thus, 98% of women had been followed up within 2 years. Time under observation for this study varied widely because of the wide range of diagnosis dates included in the study. Among women diagnosed in 1996, the maximum follow-up was 40 months. Therefore, censoring in this study was strongly related to date at diagnosis. After matching tissue blocks were ascertained, all personal identifiers were stripped from the database.

Laboratory Analysis. For genotyping for deletions in *GSTM1* and *GSTT1*, normal tissue was obtained from archival specimens. The majority of specimens (50-μm slices) were derived from normal lymph nodes (76%), with skin and breast tissue used when nodes were not available. Tissue slices were deparaffinized, and the DNA was extracted by a commercial kit (Qiagen, Chatworth, CA). Multiplex PCR was used to simultaneously amplify *GSTM1* and *GSTT1* (7), with albumin as a control gene. For *GSTM1*, primers 5′-GAACTCCCTGAAAAGCTAAAGC-3′ and 5′-GTTGGGCTCAAATATACGGTGG-3′ were used; for *GSTT1*, primers T1 (5′-TTCCTTACTGGTCCTCACATCTC-3′) and T2 (5′-TCACCGGATCATGGCCAGCA-3′) were used. The absence of amplified *GSTM1* or *GSTT1* product (in the presence of the albumin PCR product) indicated the respective null genotype for each.

Received 3/7/01; accepted 8/2/01.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

[1] This research was supported by the Arkansas Breast Cancer Research Program. C. S. and G. Y. M. were supported by fellowships from the National Center for Toxicological Research/Oak Ridge Institute for Science and Engineering.

[2] To whom requests for reprints should be addressed, at Derald H. Ruttenberg Cancer Center, Mount Sinai School of Medicine, Box 1130, One Gustave L. Levy Place, New York, NY 10029. Phone: (212) 659-5552; Fax: (212) 849-2564; E-mail: christine.ambrosone@mssm.edu.

[3] The abbreviations used are: ROS, reactive oxygen species; GST, glutathione *S*-transferase; HR, hazard ratio; CI, confidence interval.

[4] B. F. Coles, unpublished data.

Because the *GSTT1* PCR assay produces a relatively long product, there is a chance of false null *GSTT1* genotypes when archival specimens are used because of fragmented DNA from the fixed tissue samples. We therefore confirmed *GSTT1*-null results by a second assay, with forward primer 5′-CATCCCTGCCCTCACAACCA-3′ and reverse primer 5′-CTTCTGCTTTATGGTGGGGTCTG-3′ amplifying a 214-bp section of the *GSTT1* gene, with concurrent amplification of the albumin gene. The reliability of the 214-bp *GSTT1* PCR assay had been tested with DNA extracted from blood samples from a case-control study. Concordance of results from the conventional multiplex assay and the short-product *GSTT1* assay for 96 DNA samples from blood was 100%. For DNA samples extracted from paraffin blocks for the present study, the short-product *GSTT1* assay was run for all 75 samples that had null *GSTT1* results from the multiplex assay and for a randomly selected group of samples with *GSTT1*-present results. Laboratory personnel were blinded to previous results. Three samples with *GSTT1*-null results from the multiplex assay, *i.e.*, no 480-bp product, did produce a 214-bp *GSTT1* PCR product, and results from these specimens were excluded from the analyses.

Statistical Analysis. Initial analyses included the assessment of patient and tumor characteristics by *GSTM1* and *GSTT1* genotypes, using χ^2 analysis and Fisher's exact test, when appropriate. The effects of genotype on both cancer recurrence and overall survival were evaluated by the Kaplan-Meier survival function, log-rank tests for survival differences, and Cox proportional hazards models (8), with an *a priori* hypothesis that women with gene deletions for *GSTM1* and *GSTT1* would have better survival. Thus, HRs represent relative risk of death among women with null genotypes compared with those with the *GSTM1*- or *GSTT1*-present alleles. They were calculated from the Cox model first by univariate analysis, then from a multivariate model with adjustment for prognostic factors. Adjusted models included age (four categories), stage, and node status at diagnosis as stratifying variables and race (Caucasian or African-American, excluding other or unknown) as covariate. Estrogen and progesterone receptor status were also evaluated as potential confounders, but did not alter HRs for *GSTM1* and *GSTT1* genotypes and were not included in the final models. For analysis of disease-free survival, time from disease-free date to recurrence, death, or last follow-up was calculated, and adjusted HRs were estimated from the Cox model, including prognostic factors as described for analysis of overall survival. To examine the combined effects of *GSTM1* and *GSTT1* on survival and recurrence, a dummy variable was created with three categories representing presence of alleles for the following: both *GSTM1* and *GSTT1*; presence of one of the GST genes; and null status for both *GSTM1* and *GSTT1*.

RESULTS

We report here on data from 251 women who received either chemotherapy or radiation therapy (or a combination) for breast cancer. As shown in Table 1, the population was primarily Caucasian (80%) and ranged in age from 25 to 78 years. Women in this study (representative of the patient population at the Arkansas Cancer Research Center) were younger and had a more advanced stage at diagnosis than the observed distribution of breast cancer in most incident populations. Almost 75% of patients were under age 60, and >50% had nodal involvement. These characteristics could, in part, be attributable to the fact that the Arkansas Cancer Research Center is a tertiary care facility. When women were classified by genotype, 46% of Caucasians and 52% of African-Americans were *GSTM1* null. For *GSTT1*, 26% of Caucasians had the null genotype, and 40% of African-Americans carried the deletion polymorphism. Table 1 also shows the proportion of demographic and pathological characteristics by genotype status for *GSTM1* and *GSTT1*. As shown, there were no significant differences by genotype for any of these characteristics; however, allele distribution varied by race, and women with *GSTM1*- and *GSTT1*-present alleles were also more likely to be deceased ($P = 0.06$ and 0.09, respectively).

Because women included in the study were diagnosed anywhere between 1985 and 1996, there were varying periods of follow-up and censoring of data, as described above. To determine whether there was any bias among women with varying follow-up years, we compared characteristics of women followed up within 1 year of September 1999 with those living women not followed up within 1 year. There were no significant differences in age, race, stage, or genotype among women with follow-up within or more than 1 year.

Seventy-nine percent ($n = 197$) of the women in this study received chemotherapy, which in most cases was a combination of cyclophosphamide (95%), Adriamycin (76%), and 5-fluorouracil (80%), and 38% of those women also received radiation therapy. There was also a group ($n = 54$) who were treated only with radiation therapy. Disease recurrence was noted for 72 women, and 74 deaths were

Table 1 *Demographic and pathological characteristics of study participants, and distributions of characteristics by genotype for GSTM1 and GSTT1*

	All participants, *n* (%)	*GSTM1*+,[a] *n* (%)	*GSTM1*−,[b] *n* (%)	*P*[c]	*GSTT1*+,[d] *n* (%)	*GSTT1*−,[e] *n* (%)	*P*[c]
Total	251						
Age at diagnosis (years)							
<39	27 (11)	18 (14)	9 (8)	0.26[e]	19 (11)	8 (11)	0.10
40–49	86 (34)	49 (37)	37 (31)		67 (37)	19 (26)	
50–59	74 (29)	36 (27)	38 (32)		52 (29)	22 (30)	
60–69	45 (18)	23 (17)	22 (19)		25 (14)	20 (27)	
≥70	19 (8)	7 (5)	12 (10)		15 (9)	4 (5)	
Race							
African-American	50 (20)	24 (18)	26 (22)	0.43	30 (17)	20 (27)	0.06
Caucasian	201 (80)	109 (82)	92 (78)		148 (83)	53 (73)	
Vital status							
Alive	177 (71)	87 (65)	90 (76)	0.06	120 (67)	57 (78)	0.09
Deceased	74 (29)	46 (35)	28 (24)		58 (33)	16 (22)	
Stage with node status							
1	67 (27)	37 (28)	30 (25)	0.87	46 (26)	21 (29)	0.93
2 negative	49 (20)	27 (20)	22 (19)		36 (20)	13 (18)	
2 positive	79 (31)	43 (32)	36 (30)		57 (32)	22 (30)	
3	43 (17)	20 (15)	23 (20)		29 (16)	14 (19)	
4	13 (5)	6 (5)	7 (6)		10 (6)	3 (4)	
Estrogen receptor status							
Positive	150 (61)	76 (62)	74 (66)	0.17	108 (63)	41 (59)	0.55
Negative	94 (39)	56 (38)	38 (34)		65 (37)	29 (41)	
Progesterone receptor status							
Positive	110 (45)	58 (44)	52 (46)	0.74	82 (47)	28 (40)	0.29
Negative	133 (55)	73 (56)	60 (54)		90 (53)	43 (60)	

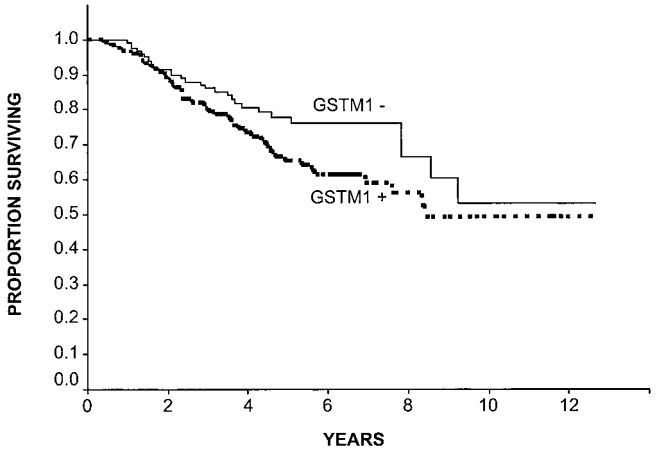
[a] *GSTM1*-present genotype.
[b] *GSTM1*-null genotype.
[c] *P* for significance of χ^2 statistic for differences between observed and expected values of selected variables by genotypes for present and null alleles. Kmc[d] *GSTT1*-present genotype.
[e] *GSTT1*-null genotype.

recorded. Median follow-up by the Tumor Registry of 177 women alive at last contact was 58 months. For women whose disease recurred (and with information on subsequent treatments), 60% received chemotherapy and 37% received radiotherapy after relapse.

Kaplan-Meier survival curves (Figs. 1 and 2) showed unadjusted relationships between *GSTM1* and *GSTT1* genotypes and survival after treatment for breast cancer. As hypothesized, null alleles for both *GSTM1* and *GSTT1* were associated with better overall survival. Cox proportional hazards models were used to estimate hazard of death and are presented in Table 2, both univariate estimates and ratios adjusted for other prognostic and possibly confounding factors. In crude models, there were nonsignificant reductions in hazard of death to 0.66 and 0.63 among women who were null for *GSTM1* and *GSTT1*, respectively. However, when models were adjusted for age, race, and stage, estimates were further reduced and strengthened (*GSTM1*, HR = 0.59, 95% CI, 0.36–0.97; *GSTT1*, HR = 0.51, 95% CI, 0.29–0.90).

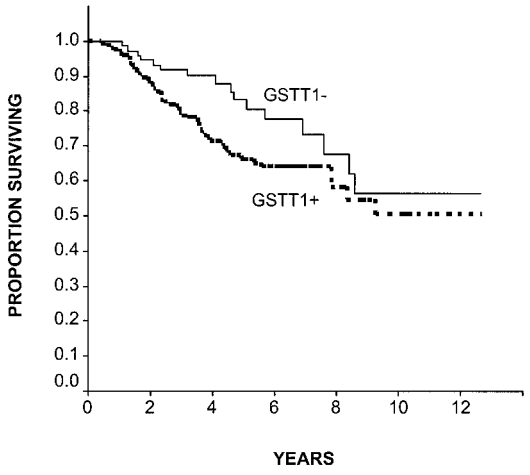
We were also interested in the combined effects that null genotypes for both *GSTM1* and *GSTT1* may have had on survival. When we tested the relationships between hazard of death and presence of *GSTM1* but not *GSTT1*, *versus GSTT1* present and *GSTM1* null, we found that there were similar associations for each genotype. Thus, we grouped either *GSTM1*- or *GSTT1*-null together as one of three categories and modeled this new combined variable on hazard of death. With the referent category those who had both *GSTM1* and *GSTT1* present, there was what appeared to be a dose-response decrease in risk with one and two null alleles (Fig. 3). Adjusted models showed slightly more than half the hazard of death among those with either M1 or T1 present, and a two-thirds reduction in risk for those who were null for both genotypes (Table 2).

Relationships between genotypes and recurrence were also evaluated and are shown in Table 3. *GSTM1*- and *GSTT1*-null genotypes, considered separately or in combined categories, were again associated with less recurrence. HRs for recurrence-free survival were similar to those for overall survival, although relationships with *GST* genotype were somewhat attenuated, perhaps because women who were never disease-free had to be excluded from this analysis, thus reducing sample size.



Years from diagnosis	0	2	4	6	8	10	12
GSTM1-	118	105	66	36	14	5	0
GSTM1+	133	116	66	44	18	7	1
Totals	251	221	132	80	32	12	1

Fig. 1. Kaplan-Meier function for overall survival among women treated for breast cancer, by *GSTM1* genotype. Test for survival difference by log-rank method: *GSTM1*, $P = 0.09$.



Years from diagnosis	0	2	4	6	8	10	12
GSTT1-	73	67	41	23	11	4	0
GSTT1+	178	154	91	57	21	8	1
Totals	251	221	132	80	32	12	1

Fig. 2. Kaplan-Meier function for overall survival among women treated for breast cancer, by *GSTT1* genotype. Test for survival difference by log-rank method: *GSTT1*, $P = 0.09$.

Although relationships between genotypes and survival appeared clear and strong, we conducted further analyses to explore whether those relationships were possibly the result of other demographic or prognostic factors or whether they varied markedly by type of treatment given. Therefore, we stratified data separately on a number of the variables of potential importance, including stage at diagnosis and treatment received, and examined genotype and survival within each category. As shown in Table 4, there appeared to be some difference in relationships between genotype and disease-free survival depending on stage at diagnosis, although the numbers were small and the confidence intervals wide, so it is likely that the estimates were unstable. However, for *GSTM1*, it appeared that the relationship was important only among women with stage 1 and 2 disease. Perhaps in women with more advanced, aggressive disease, the impact of variability in response to treatment agents may have been too subtle and the disease severity outweighed any modifying impact of genotype. However, in relation to *GSTT1* genotype, the effect was greatest among women with advanced disease, although there were only five women with the null genotype who did not survive. Although treatment given (chemotherapy or radiation therapy) did not appear to alter relationships between *GSTT1* and genotype, among women who received only radiation therapy, there were no deaths for those with *GSTM1*-null genotypes, whereas 21% of those with *GSTM1* present did not survive.

DISCUSSION

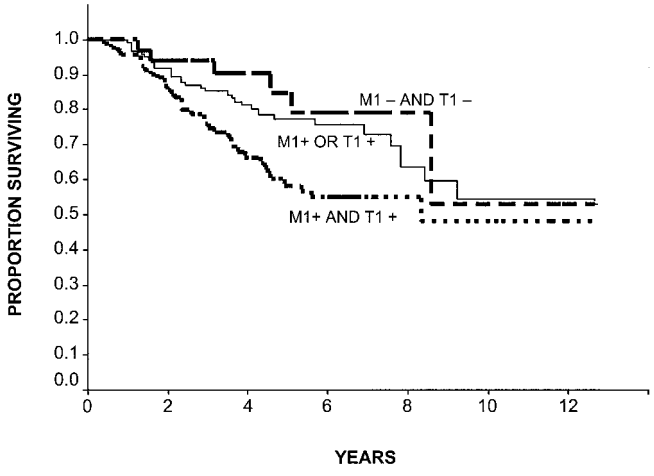
In this study, we found that the null genotypes for *GSTM1* and *GSTT1* and, particularly, the combined impact of deletion of both genes significantly reduced hazard of death among women who received treatment for breast cancer. Women who were null for either *GSTM1* or *GSTT1* had half the hazard of death than those with at least one allele, and, in comparison with those with both *GSTM1* and *GSTT1* present, women with both null genotypes had one-third the

Table 2 *Associations between GSTM1 and GSTT1 genetic polymorphisms and survival after treatment for breast cancer*

Test for survival difference by log-rank method: *GSTM1*, $P = 0.09$; *GSTT1*, $P = 0.07$; combined categories, $P = 0.03$.

Genotype	Total cases (*n*)	Number of deaths, *n* (%)	HR[a] (95% CI)	HR[b] (95% CI)
GSTM1				
Present	133	46	1.0 (Reference)	1.0 (Reference)
Null	118	28	0.66 (0.42–1.07)	0.59 (0.36–0.97)
GSTT1				
Present	178	58	1.0 (Reference)	1.0 (Reference)
Null	73	16	0.59 (0.34–1.01)	0.51 (0.29–0.90)
GSTM1 and *GSTT1* combined				
M1 and T1 present	93 (37)	36 (22)	1.0 (Reference)	1.0 (Reference)
M1 or T1 present	125 (50)	32 (37)	0.61 (0.38–0.99)	0.54 (0.33–0.86)
Both M1 and T1 null	33 (13)	6 (11)	0.43 (0.18–1.02)	0.28 (0.11–0.70)

[a] HRs for overall survival from Cox proportional hazards model, unadjusted.
[b] HRs for overall survival from Cox proportional hazards model, adjusted for age, race, stage at diagnosis, and node status.



Years from diagnosis	0	2	4	6	8	10	12
M1 – &T1 –	33	30	20	10	3	2	0
M1+ or T1+	125	112	67	39	19	5	0
M1 + &T1 +	93	79	45	31	10	5	1
Totals	251	221	132	80	32	12	1

Fig. 3. Kaplan-Meier function for overall survival among women treated for breast cancer, by combined genotypes for *GSTM1* and *GSTT1*. Test for survival difference by log-rank method: combined categories, $P = 0.04$.

risk of death following treatment. These results are consistent with our hypotheses and could be explained by the role of GSTs in the removal of reactive products resulting from chemotherapy and radiation therapy.

The majority of women in this study were treated with a combination of cyclophosphamide, Adriamycin, and 5-fluorouracil, and a large proportion of them also received radiation therapy, either alone or in combination with chemotherapy.

It is known that the mechanism of cytotoxicity with radiation therapy is through the generation of ROS (2). Radiation therapy may eradicate cancer cells either directly through effects on the target molecules or indirectly through intermediate radiation products (2). When water, the most abundant intra- and extracellular material, is exposed to ionizing radiation, decomposition occurs, through which a variety of ROS, including superoxide radicals, hydrogen peroxide, and hydroxyl radicals, are generated (9). These reactive species can damage cells, proteins, and DNA or interact with other cellular molecules, producing secondary oxidation products, reactive molecules that contribute to cytotoxicity through the same mechanisms.

Generation of ROS is part of the cytotoxic activity of chemotherapy agents as well. Numerous clinical studies have shown that patients treated with a wide range of cytotoxic agents have marked increases in lipid peroxidation products (10–13). Decreases in vitamin E concentrations were also noted after treatment (13). In the population in this study, women were treated primarily with cyclophosphamide, Adriamycin, and 5-fluorouracil. There are data that all of the agents used, but particularly cyclophosphamide and Adriamycin, result in lipid peroxidation and generation of ROS. Numerous studies have noted that administration of cyclophosphamide or its metabolite, acrolein, to rats results in an increase in lipid peroxidation products, such as malondialdehyde (14–16). Cyclophosphamide exposure also results in concomitant decreases in glutathione (17) and the endogenous antioxidants superoxide dismutase and glutathione peroxidase (16). The mechanism of by which cyclophosphamide kills tumor cell through ROS is further demonstrated by rodent data showing that the lung injury associated with treatment with cyclophosphamide is attributable to its ability to generate free radicals (15, 18). Exposure to Adriamycin, an antineoplastic antibiotic widely used in breast cancer treatment, like other anthracyclines can result in the formation of

Table 3 *Risk of recurrence among 251 women treated for breast cancer in relation to genetic polymorphisms in GSTM1 and GSTT1*

	Recurrence status				
	Disease-free, *n* (%)	Recurred, *n* (%)	Never disease-free,[a] *n* (%)	Crude HR[b] (CI)	Adjusted HR[c] (CI)
GSTM1					
Present	86 (51)	43 (60)	4 (44)	1.0	1.0
Null	84 (49)	29 (40)	5 (66)	0.72 (0.47–1.10)	0.67 (0.43–1.03)
GSTT1					
Present	118 (69)	51 (71)	9 (100)	1.0	1.0
Null	52 (31)	21 (29)	0 (0)	0.82 (0.51–1.31)	0.68 (0.42–1.12)
GSTM1 and *GSTT1* combined					
M1 and *T1* present	59 (35)	30 (42)	4 (44)	1.0	1.0
M1 or *T1* present	86 (51)	34 (47)	5 (66)	0.74 (0.48–1.14)	0.65 (0.41–1.02)
Both *M1* and *T1* null	25 (15)	8 (11)	0 (0)	0.60 (0.29–1.24)	0.47 (0.22–1.00)

[a] Subjects who were never disease free are omitted from calculation of hazard ratio for recurrence-free survival.
[b] HRs for recurrence-free survival from Cox proportional hazards model, unadjusted, with 95% CI.
[c] HRs for recurrence-free survival from Cox proportional hazards model, adjusted for age, race, stage at diagnosis, and node status, with 95% CI.

Table 4 *Associations between genotypes and disease-free survival, stratified by stage and treatment received*

	Deaths/At risk, *n* (%)		
	Present	Null	HR (95% CI)
GSTM1			
Stage at diagnosis			
1 and 2	30/107 (28)	14/88 (16)	0.50 (0.26–0.97)
3 and 4	16/26 (62)	14/30 (47)	0.98 (0.41–2.34)
Treatment			
Chemotherapy	30/69 (43)	16/53 (30)	0.51 (0.25–1.03)
Radiation	6/29 (21)	0/25 (0)	Undefined: no deaths, *GSTM1*-null
GSTT1			
Stage at diagnosis			
1 and 2	33/139 (28)	11/56 (16)	0.77 (0.38–1.54)
3 and 4	25/39 (62)	5/17 (47)	0.17 (0.04–0.74)
Treatment			
Chemotherapy	35/86	11/36	0.59 (0.26–1.39)
Radiation	5/35	1/19	0.63 (0.06–6.31)

quinone-mediated free radicals (19). Adriamycin-generated free radicals have the capacity to cause oxidative damage and cytotoxicity (20). Lipid peroxides resulting from doxorubicin can break down to yield hydroxyalkenals, which are substrates for glutathione-conjugating isozymes (21). The fact that its tumor cell-killing mechanism is through oxidative stress is demonstrated by data showing that Adriamycin's cardiotoxicity is a result of the production of ROS (22, 23), which are presumably acting on tumor cells as well. As stated above, both GSTM1 and GSTT1 have been shown to have activity toward lipid hydroperoxides, and clearly their activity in the removal of lipid hydroperoxides may be key to DNA damage and cytotoxicity.

There have been few studies of GST genetic polymorphisms and survival. For the most part, prior studies of treatment efficacy and the GSTs have been based on studies of phenotype in tumor tissue, and these have primarily been in relation to GST π. GST π and μ are reported present in normal and tumor breast tissue (24, 25), and we have noted that GSTT1 is expressed in breast tumor tissue as well.[5] However, the impact of GST genotype may also be important for hepatic detoxification and/or systemic effects via the presence of the GSTs in lymphocytes and erythrocytes (26). In fact, studies relating levels of GSTs in peripheral blood to response to therapy support this notion (27, 28).

Two studies of similar size investigated associations between GST genetic polymorphisms and survival with ovarian cancer. In one study (29), there was no effect for *GSTM1* or *GSTT1* genotype alone, but the combined null genotypes for *GSTM1* and *GSTT1* were associated with poorer survival. A recent study, however, reported no association between *GSTM1* genotype and ovarian cancer survival (30). In studies of hematopoietic cancers, reduced risk of disease recurrence was noted among children with acute lymphoblastic leukemia who had alleles encoding no or lower activity for GSTM1, -P1, and -T1 (31). Davies *et al.* (32) reported increased therapy-related toxicity among the *GSTT1*-null patients with acute myelocytic leukemia. These findings support the hypothesis that patients with *GSTT1*-null genotypes have reduced detoxification of therapeutic agents and, in the case of high-dose therapy for acute myelocytic leukemia, worse outcomes. We believe that, in the present study of primary breast cancer patients, the better outcomes that were observed among *GSTT1*-null patients who were not treated with high-dose therapy can be explained by the increased efficacy of treatment. We recently found that women with the less active variant for *GSTP1* had improved survival after breast cancer treatment (6), possibly because of the role of GSTP1 in metabolism of cyclophosphamide. Contrary to observations reported here, a hospital-based study (33) reported reduced survival among breast cancer patients with the *GSTM1*-null genotype. However, whether patients received adjuvant therapy was not reported, and HRs were not adjusted for other prognostic factors.

In our data, the effect of the *GSTM1*- and *GSTT1*-null genotypes on survival after treatment for breast cancer was evident in the entire population. Stratification by stage at diagnosis and treatment received, however, appeared to alter associations, with a much stronger effect of the *GSTM1*-null genotype among women who received only radiation, although the association was still evident for those treated with chemotherapy. The effects of *GSTM1* and *GSTT1* also appeared to vary by stage at diagnosis; whereas the *GSTT1*-null genotype reduced the risk of death in women with advanced disease, the inverse associations with the null genotype for *GSTM1* were apparent only with stage 1 and 2 disease. Interestingly, in a small study of women with advanced breast cancer who received chemotherapy (34), *GSTM1* genotype had no effect on survival, which is consistent with our findings when stratified by stage of diagnosis. However, the numbers in these stratified analyses were small, and risk estimates were likely to be unstable. Hence, these relationships should be further explored with specific emphasis on stage at diagnosis and treatment received.

The patient population was also heterogeneous in other well-established prognostic factors, including age and hormone receptor status. Relationships between genotype and prognosis were evaluated within these categories by stratified analyses, with no differences noted between groups (data not shown). Furthermore, in the absence of evidence of associations between genotype and other prognostic factors, it is likely that genotypes are randomly distributed (regardless of, *e.g.,* tumor stage and grade and estrogen receptor and progesterone receptor status). This nondifferential distribution is also likely to apply to other tumor tissue characteristics that may have prognostic importance, such as p53, Bcl-2, and ErbB2 overexpression, as well as proteins associated with multidrug resistance. With evidence that genotype is independent of stage, grade, and hormone receptor status, there is little theoretical rationale to support the possibility that associations between genotype and survival would be differentially impacted by other prognostic factors, such as tumor tissue characteristics. There is also the possibility that women with *GSTM1*- and *GSTT1*-present genotypes, being more resistant to therapy, were likely to receive higher doses of chemotherapy and/or radiation. Unfortunately, we do not have sufficient data to address this question; however, if women with those genotypes received more intensive treatments than those with null alleles, the associations we observed would be attenuated, and true relationships would actually be stronger than those reported.

Distribution of the null genotype for both *GSTM1* and *GSTT1* was higher in our patient population than in most populations for controls, as well as for cases, particularly for African-Americans. For most studies of *GSTM1* and *GSTT1*, the null allele is present in ~50% and 15–20% of Caucasians of European descent, respectively. In the Carolina Breast Study (35), 51 and 52% of Caucasian cases and controls had the *GSTM1* deletion, and 15 and 16% were null for *GSTT1*. For African-Americans, 25 and 28% of cases and controls were null for *GSTM1*, and 20 and 17% were null for *GSTT1*. The high proportion of African-American women who are null for *GSTM1* and *GSTT1* may be attributable to chance, or it may indicate that this genotype increases risk of breast cancer in our study population. We are unable to explore this issue further, however, in the absence of an appropriate control group.

As stated above, most studies in the past have evaluated GST polymorphisms in relation to cancer risk, rather than prognosis. For the most part, studies have found no increased risk for breast cancer with null genotypes for *GSTM1* and/or *GSTT1* (reviewed in Ref. 36),

[5] B. F. Coles, C. Sweeney, L. Joseph, M. Y. Fares, and C. B. Ambrosone, unpublished data.

although there have been some positive findings (37, 38). Nor does there appear to be any association between genotype and stage at diagnosis or risk of breast cancer according to disease stage (35, 39). However, one of the earlier studies of *GSTM1* and breast cancer indicated that the null genotype could be related to survival because of its association with risk in women with prevalent, but not incident breast cancer (40).

In summary, we found that genetic polymorphisms in GSTs M1 and T1, known to be involved in response to ROS and products of lipid peroxidation resulting from chemo- and radiation therapy, were associated with significantly reduced hazard of death and risk of recurrence following treatment for breast cancer. Women with null genotypes for both *GSTM1* and *GSTT1* had one-third the hazard of death than those with alleles for both genes present. It has been suggested that the use of genomics in therapeutic decision-making may play an important role in the clinic, with microarrays specific for metabolic treatment pathways used for individualized dosing in the future (41). Findings such as ours, if corroborated, could be useful in tailoring therapeutic regimens based on patient genotypes predictive of increased efficacy and decreased toxicity among patients being treated for cancer.

ACKNOWLEDGMENTS

We thank Christopher Wren for technical assistance and Linda Erkman, tumor registrar at the Arkansas Cancer Research Center, for assistance with Registry data.

REFERENCES

1. Weijl, N. I., Cleton, F. J., and Osanto, S. Free radicals and antioxidants in chemotherapy-induced toxicity. Cancer Treat. Rev., *23:* 209–240, 1997.
2. Hellman, S. Principles of radiation therapy. *In:* V. T. DeVita, S. Hellman, and S. A. Rosenberg (eds.), Cancer: Principles and Practice of Oncology, Ed. 4, pp. 248–275. Philadelphia: J. B. Lippincott Co., 1993.
3. La Torre, F., Orlando, A., Silipigni, A., Giacobello, T., Pergolizzi, S., and Aragona, M. Increase of oxygen free radicals and their derivatives in chemo- and radiation treated neoplasm patients. Minerva Med., *88:* 121–126, 1997.
4. Hayes, J. D., and McLellan, L. I. Glutathione and glutathione-dependent enzymes represent a co-ordinately regulated defence against oxidative stress. Free Radic. Res., *31:* 273–300, 1999.
5. Hurst, R., Bao, Y., Jemth, P., Mannervik, B., and Williamson, G. Phospholipid hydroperoxide glutathione peroxidase activity of human glutathione transferases. Biochem. J., *332:* 97–100, 1998.
6. Sweeney, C., McClure, G. Y., Fares, M. Y., Stone, A., Coles, B. F., Thompson, P. A., Korourian, S., Hutchins, L. F., Kadlubar, F. F., and Ambrosone, C. B. Association between survival after treatment for breast cancer and glutathione *S*-transferase P1 Ile105Val polymorphism. Cancer Res., *60:* 5621–5624, 2000.
7. Arand, M., Muhlbauer, R., Hengstler, J., Jager, E., Fuchs, J., Winkler, L., and Oesterling, J. E. A multiplex polymerase chain reaction protocol for the simultaneous analysis of the glutathione *S*-transferase GSTM1 and GSTT1 polymorphisms. Anal. Biochem., *236:* 184–186, 1996.
8. Clayton, D., and Hills, M. Statistical Models in Epidemiology. Oxford: Oxford University Press, 1993.
9. Sun, J., Chen, Y., Li, M., and Ge, Z. Role of antioxidant enzymes on ionizing radiation resistance. Free Radic. Biol. Med, *24:* 586–593, 1998.
10. Look, M. P., and Musch, E. Lipid peroxides in the polychemotherapy of cancer patients. Chemotherapy, *40:* 8–15, 1994.
11. Subramaniam, S., Shyama, S., Jagadeesan, M., and Shyamala Devi, C. S. Oxidant and antioxidant levels in the erythrocytes of breast cancer patients treated with CMF. Med. Sci. Res., *21:* 70–80, 1993.
12. Faure, H., Coudray, C., Mousseau, M., Ducros, V., Douki, T., Bianchini, F., Cadet, J., and Favier, A. 5-Hydroxymethyluracil excretion, plasma TBARS and plasma antioxidant vitamins in Adriamycin-treated patients. Free Radic. Biol. Med., *20:* 979–983, 1996.
13. Faber, M., Coudray, C., Hida, H., Mousseau, M., and Favier, A. Lipid peroxidation products, and vitamin and trace element status in patients with cancer before and after chemotherapy, including Adriamycin. A preliminary study. Biol. Trace Elem. Res., *47:* 117–123, 1995.
14. Sulkowska, M., Sulkowski, S., Skrzydlewska, E., and Farbiszewski, R. Cyclophosphamide-induced generation of reactive oxygen species. Comparison with morphological changes in type II alveolar epithelial cells and lung capillaries. Exp. Toxicol. Pathol., *50:* 209–220, 1998.
15. Patel, J. M. Metabolism and pulmonary toxicity of cyclophosphamide. Pharmacol. Ther., *47:* 137–146, 1990.
16. Kaya, H., Oral, B., Ozguner, F., Tahan, V., Babar, Y., and Delibas, N. The effect of melatonin application on lipid peroxidation during cyclophosphamide therapy in female rats. Zentralbl. Gynakol., *121:* 499–502, 1999.
17. Berrigan, M. J., Struck, R. F., and Gurtoo, H. L. Lipid peroxidation induced by cyclophosphamide. Cancer Biochem. Biophys., *9:* 265–270, 1987.
18. Venkatesan, N., and Chandrakasan, G. Modulation of cyclophosphamide-induced early lung injury by curcumin, an anti-inflammatory antioxidant. Mol. Cell Biochem., *142:* 79–87, 1995.
19. Doroshow, J. H., and Locker, G. Y. Role of oxygen radical formation in anthracycline cardiac toxicity. *In:* F. M. Muggia, C. W. Young, and S. K. Carter (eds.), Anthracycline Antibiotics in Cancer Therapy, p. 317. The Hague: Martinus Nijhoff, 1982.
20. Doroshow, J. H., Akman, S., Chu, F. F., and Esworthy, S. Role of the glutathione-glutathione peroxidase cycle in the cytotoxicity of the anticancer quinones. Pharmacol. Ther., *47:* 359–370, 1990.
21. Sellin, S., Holmquist, B., Mannervik, B., and Vallee, B. L. Oxidation and reduction of 4-hydroxyalkenals catalyzed by isozymes of human alcohol dehydrogenase. Biochemistry, *30:* 2514–2518, 1991.
22. Ravid, A., Rocker, D., Machlenkin, A., Rotem, C., Hochman, A., Kessler-Icekson, G., Liberman, U. A., and Koren, R. 1,25-Dihydroxyvitamin D3 enhances the susceptibility of breast cancer cells to doxorubicin-induced oxidative damage. Cancer Res., *59:* 862–867, 1999.
23. DeAtley, S. M., Aksenov, M. Y., Aksenova, M. V., Jordan, B., Carney, J. M., and Butterfield, D. A. Adriamycin-induced changes of creatine kinase activity in vivo and in cardiomyocyte culture. Toxicology, *134:* 51–62, 1999.
24. Alpert, L. C., Schecter, R. L., Berry, D. A., Melnychuk, D., Peters, W. P., Caruso, J. A., Townsend, A. J., and Batist, G. Relation of glutathione *S*-transferase α and μ isoforms to response to therapy in human breast cancer. Clin. Cancer Res., *3:* 661–667, 1997.
25. Murray, G. I., Weaver, R. J., Paterson, P. J., Ewen, S. W., Melvin, W. T., and Burke, M. D. Expression of xenobiotic metabolizing enzymes in breast cancer. J. Pathol., *169:* 347–353, 1993.
26. Wiencke, J. K., Pemble, S., Ketterer, B., and Kelsey, K. T. Gene deletion of glutathione *S*-transferase θ: correlation with induced genetic damage and potential role in endogenous mutagenesis. Cancer Epidemiol. Biomark. Prev., *41:* 253–258, 1995.
27. Hida, T., Kuwabara, M., Ariyoshi, Y., Takahashi, T., Sugiura, T., Hosoda, K., Niitsu, Y., and Ueda, R. Serum glutathione *S*-transferase-π level as a tumor marker for non-small cell lung cancer. Cancer (Phila.), *73:* 1377–1382, 1994.
28. Hirata, S., Odajima, T., Kohama, G., Ishigaki, S., and Niitsu, Y. Significance of glutathione *S*-transferase π as a tumor marker in patients with oral cancer. Cancer (Phila.), *70:* 2381–2387, 1992.
29. Howells, R. E., Redman, C. W., Dhar, K. K., Sarhanis, P., Musgrove, C., Jones, P. W., Alldersea, J., Fryer, A. A., Hoban, P. R., and Strange, R. C. Association of glutathione *S*-transferase GSTM1 and GSTT1 null genotypes with clinical outcome in epithelial ovarian cancer. Clin. Cancer Res., *4:* 2439–2445, 1998.
30. Lallas, T. A., McClain, S. K., Shahin, M. S., and Buller, R. E. The glutathione *S*-transferase M1 genotype in ovarian cancer. Cancer Epidemiol. Biomark. Prev., *9:* 587–590, 2000.
31. Stanulla, M., Schrappe, M., Brechlin, A. M., Zimmermann, M., and Welte, K. Polymorphisms within glutathione *S*-transferase genes (GSTM1, GSTT1, GSTP1) and risk of relapse in childhood B-cell precursor acute lymphoblastic leukemia: a case-control study. Blood, *95:* 1222–1228, 2000.
32. Davies, S. M., Buckley, J. D., Robinson, L. L., Tjoa, T., Woods, W. G., Radloff, G. A., Ross, J. A., and Perentesis, J. P. Glutathione *S*-transferase polymorphisms and outcome of chemotherapy in childhood AML. Blood, *94:* S1, 1999.
33. Nedelcheva, K., V, Andersen, T. I., Erikstein, B., Geitvik, G., Skovlund, E., Nesland, J. M., and Borresen-Dale, A. L. Single tube multiplex polymerase chain reaction genotype analysis of GSTM1, GSTT1 and GSTP1: relation of genotypes to TP53 tumor status and clinicopathological variables in breast cancer patients. Pharmacogenetics, *8:* 441–447, 1998.
34. Lizard-Nacol, S., Coudert, B., Colosetti, P., Riedinger, J-M., Fargeot, P., and Brunet-Lecomte, P. Glutathione *S*-transferase M1 null genotype: lack of association with tumour characteristics and survival in advanced breast cancer. Breast Cancer Res. Treat., *1:* 81–87, 1999.
35. Millikan, R. C., Pittman, G., Tse, C-K., Savitz, D., Newman, B., and Bell, D. A. Glutathione *S*-transferases M1, T1, and P1, and breast cancer. Cancer Epidemiol. Biomark. Prev., *9:* 567–574, 2000.
36. Dunning, A. M., Healey, C. S., Pharoah, P. D., Teare, M. D., Ponder, B. A. J., and Easton, D. F. A systematic review of genetic polymorphisms and breast cancer risk. Cancer Epidemiol. Biomark. Prev., *8:* 843–854, 1999.
37. Helzlsouer, K. J., Selmin, O., Huang, H-Y., Strickland, P. T., Hoffman, S., Alberg, A. J., Watson, M., Comstock, G. W., and Bell, D. A. Association between glutathione *S*-transferase M1, P1, and T1 genetic polymorphisms and development of breast cancer. J. Natl. Cancer Inst. (Bethesda), *90:* 512–518, 1998.
38. Mitrunen, K., Jourenkova, N., Kataja, V., Eskelinen, M., Kosma, V-M., Benhamou, S., Vainio, H., Uusitupa, M., and Hirvonen, A. Glutathione *S*-transferase M1, M3, P1, and T1 genetic polymorphisms and susceptibility to breast cancer. Cancer Epidemiol. Biomark. Prev., *10:* 229–236, 2001.
39. Shea, T., Claflin, G., Comstock, K., Sanderson, B., Burstein, N., Keenan, E., Mannervik, B., and Henner, W. D. Glutathione transferase activity and isoenzyme composition in primary human breast cancers. Cancer Res., *50:* 6848–6853, 1990.
40. Kelsey, K., Hankinson, S., Colditz, G., Springer, K., Garcia-Closas, M., Spiegelman, D., Manson, J. A. E., Garland, M., Stampfer, M. J., Willett, W., Speizer, F. E., and Hunter, D. Glutathione *S*-transferase class μ deletion polymorphism and breast cancer: results from prevalent *versus* incident cases. Cancer Epidemiol. Biomark. Prev., *9:* 567–573, 1997.
41. Evans, W. E., and Relling, M. V. Pharmacogenomics: translating functional genomics into rational therapeutics. Science (Wash. DC), *286:* 487–491, 1999.