Polymorphisms in *GSTP1*, *GSTM1*, and *GSTT1* and Susceptibility to Colorectal Cancer[1]

Mark Welfare,[2] A. Monesola Adeokun, Margaret F. Bassendine, and Ann K. Daly

Departments of Pharmacological Sciences [M. W., A. M. A., A. K. D.] and Medicine [M. W., M. F. B.], University of Newcastle upon Tyne, Newcastle upon Tyne NE298NH, England

Abstract

Polymorphisms in glutathione *S*-transferases (GSTs) may predispose to colorectal cancer through deficient detoxification of environmental carcinogens, although previous results are conflicting. A study with 178 matched case-control pairs was conducted to determine the effect of the *GSTT1* and *GSTM1* null genotypes and polymorphisms in *GSTP1* on colorectal cancer susceptibility. In a secondary analysis, we examined interactions between genotypes and with the *N-acetyltransferase 2* (*NAT2*) genotype. Heterogeneity by age, sex, site, and stage of cancer was also examined. No effect of any genotype for *GSTM1*, *GSTT1*, or *GSTP1* on colorectal cancer susceptibility was detected. Secondary end points showed that individuals with both the *GSTT1* null and *NAT2* slow genotypes combined appeared to be at increased risk of colorectal cancer (odds ratio = 2.33; 95% confidence interval, 1.1–5.0). We conclude that GST polymorphisms alone do not predispose to colorectal cancer in northeast England. We also observed possible effects of the *GSTT1* null genotype on the age and stage at presentation, and these, together with the findings of an apparent interaction with *NAT2* genotypes, need to be confirmed in further studies.

Introduction

GSTs[3] are a large and diverse family of enzymes, and in humans, there are at least 13 GST enzymes belonging to five families, namely α (GSTA), μ (GSTM), π (GSTP), σ (GSTS), and θ (GSTT; Refs. 1 and 2). GSTs detoxify diverse electrophiles, including carcinogens, chiefly by conjugating them with glutathione. Heterocyclic amines are carcinogens that have been implicated as a potential cause of colorectal cancer in humans and that have also been shown to be detoxified by GSTs (3). In addition, foods that are known to induce the expression of GSTs are also thought to be protective against colorectal cancer (4). If it could be confirmed that GSTs are protective against colorectal cancer, it might be possible to identify individuals at high risk of this disease or to manipulate the expression of GSTs to prevent colorectal cancer by either dietary or pharmacological means.

One method of investigating the protective role of GSTs has been to study the effect of polymorphisms in GST genes on susceptibility to colorectal cancer. Functional polymorphisms are known for three of the human genes, namely *GSTM1*, *GSTT1*, and *GSTP1* (5–8). For both *GSTM1* and *GSTT1*, the variant allele is a deletion of the gene, and individuals who are homozygous for the deleted allele are said to possess the "null" genotype and do not express the enzyme at all. For *GSTP1*, two genetic polymorphisms are known, Ile-105→Val, resulting from an A→G transition at base 1578, and Ala-114→Val, resulting from a C→T transition at base 2293 (8). The *GSTP1*A* allele possesses Ile-105 and Ala-114 and *GSTP1*B* has Val-105 and Ala-114, whereas *GSTP1*C* has both Val-105 and Val-114. Site-directed mutagenesis has been used to show the functional significance of the Ile-105→Val polymorphism. Alleles with Ile-105 have greater activity with 3,4-dichloro-1-nitrobenzene, whereas those with the Val-105 allele have greater activity with ethacrynic acid and bromosulfophthaein (8, 9).

Five published studies have examined the relationship between the *GSTM1* null genotype and susceptibility to colorectal cancer or colorectal polyps (10–14). Only the study by Zhong *et al.* (10) found increased risk associated with the null genotype, particularly for cancers of the proximal colon. Three studies have examined whether possession of the null genotype for *GSTT1* confers susceptibility to colorectal cancer (11–13). Only the largest study, with 148 cases and 577 controls, demonstrated an association between the null genotype and colorectal cancer, with an OR of 1.88 (95% CI, 1.28–2.77; Ref. 13). Two studies have examined the frequency of the Ile-105→Val *GSTP1* polymorphism, and one the frequency of the Ala-114→Val *GSTP1* polymorphism in colorectal cancer; neither study reported any association (15, 16).

The enzyme NAT2 is responsible for the polymorphism in the metabolism of drugs such as isoniazid (17). Individuals can be classified as fast or slow acetylators using metabolic probes such as sulfamethazine (phenotyping) or by analyzing sequence polymorphisms in the *NAT2* gene (genotyping). The fast acetylator phenotype of NAT2 has been reported to be associated with susceptibility to colorectal cancer (18, 19), although studies that classified acetylator status using genotyping techniques have mostly shown no overall association (20–22). Recently several studies have that shown that the risk in fast acetylators appears to be influenced by their consumption of dietary heterocyclic amines (21, 23).

The main hypotheses investigated in this paper were that polymorphisms in any of the *GSTM1*, *GSTT1*, or *GSTP1* genes predispose to colorectal cancer. The secondary questions investigated were whether there was any interaction between various combinations of *GSTP1*, *GSTT1*, *GSTM1*, and *NAT2* genotypes.

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[1] This study was funded by the North of England Cancer Research Campaign.
[2] To whom requests for reprints should be addressed, at Academic Department, North Tyneside General Hospital, Rake Lane, North Shields NE29 8NH, England. Phone: 44-191-2932576; Fax: 44-191-2932585; E-mail: m.r.welfare@ncl.ac.uk.
[3] The abbreviations used are: GST, glutathione *S*-transferase; OR, odds ratio; CI, confidence interval; NAT2, *N*-acetyltransferase 2.

Materials and Methods

This study was a case-control study using a matched pair design so that biases due to age, sex, and area of residence were minimized. Power calculation, assuming that the *GSTM1* null genotype would be present in ~45% of controls, showed that, using 1:1 matching, 160 cases were required for the study to have 90% power to detect an OR of 2.0 for the *GSTM1* null genotype at the 0.05 significance level. For the *GSTT1* polymorphism, 155 cases were required to detect an OR of 2.0 with 80% power, assuming that the population frequency of *GSTT1* null genotype would be 20%. At the time the study was designed, the *GSTP1* polymorphisms were not known, so no power calculation was made.

All patients with histologically proven colorectal cancer diagnosed in the Newcastle and North Tyneside health district (covering an area ~30 × 15 miles) in the time period December 1994–September 1995 were invited to participate if they did not meet any exclusion criteria. The main exclusion criteria were the inability to give informed consent and cancers occurring in inherited syndromes or in patients with colitis. A total of 230 cases that matched the inclusion criteria were identified, and 201 cases agreed to participate (87% recruitment). Age- and sex-matched community controls were recruited for each case. They were identified from the records of the general practitioner (primary care physician) that the matched case usually attended in an attempt to match for social circumstances. Three potential controls were identified for each case but because there was a participation rate of ~50% among controls only 178 matched case-control pairs were actually recruited (77% of the original cases). The median age of the 178 matched case and controls was identical (69 years), and 58% of the cases and controls were men. The cases for whom no control was found were younger than those for whom a control was found (59 *versus* 69 years, $P = 0.01$), but the sex distribution was not significantly different.

The study received approval from the Newcastle and North Tyneside Ethics Committee, and all subjects gave informed, written consent.

The *GSTM1* null genotype was detected using the method of Zhong *et al.* (24), and the *GSTT1* null genotype was detected using a multiplex PCR method, as described by Chenevix-Trench *et al.* (11) using the *GSTT1* primers described by Pemble *et al.* (7).

The A_{1578}→G substitution, which is present in the *GSTP1*B* and *GSTP1*C* alleles and which gives rise to the base substitution of valine for isoleucine at position 105, was detected by PCR followed by digestion with the restriction enzyme *Alw*261. The primers were 5′-GGCTCTATGGGAAGGACCAGCAGG-3′ (1323–1346 bp) and 5′-GCACCTCCATCCAGAAACTGGCG-3′ (1746–1768 bp), and the PCR was carried out using the general methods described previously (25) in a total volume of 50 μl with 30 cycles of 1 min at 94°C, 1 min at 66°C, and 2 min at 72°C. A product of 445 bp was obtained, and a 20-μl aliquot was digested with 1 unit of *Alw*261. The *GSTP1*A* allele was indicated by bands of ~330 and 115 bp when analyzed by agarose gel electrophoresis, whereas the *GSTP1*B* and *GSTP1*C* alleles had an additional site for the enzyme and were indicated by the presence of bands of 270 and 115 bp. A third band of 60 bp was not visible under the electrophoresis conditions.

The C_{2293}→T substitution is present only in the *GSTP1*C* allele and gives rise to a substitution of valine for alanine at position 114 and was detected by PCR followed by digestion with the restriction enzyme *Aci*I. The PCR primers were 5′-CAGCAGAGGCAGCGTGTGTGC-3′ (1851–1871 bp) and 5′-CCCACAATGAAGGTCTTGCCTCC-3′ (2367–2390 bp), and the PCR was carried out using the general methods described previously (25) in a total volume of 50 μl with 30 cycles of 1 min at 94°C, 1 min at 64°C, and 2 min at 72°C. A product of 539 bp was obtained, and a 20-μl aliquot was digested with 1 unit of *Aci*I. The *GSTP1*A* and *GSTP1*B* alleles were indicated by bands of 365 and 120 bp on agarose gel electrophoresis (a third band of ~80 bp is not visible), but the *GSTP1*C* allele had lost one of the two *Aci*I sites and was indicated by a single detectable band of 485 bp. The *GSTP1* genotype was allocated on the assumption that the C_{2293}→T substitution is tightly linked to the A_{1578}→G substitution, as reported by Harries *et al.* (15). The *NAT2* genotype was determined as described previously (21).

There were four primary end points (the frequency of null *GSTT1* and *GSTM1* and the frequency of the two polymorphisms of *GSTP1* in cases and controls). There were six main secondary analyses of the combination genotypes between *GSTM1*, *GSTT1*, *GSTP1*, and *NAT2*. Because other authors had suggested that there may be differences in the genotype pattern at different ages or sites or stage of tumor, the frequency of the genotypes was also examined by sex, in three age subgroups, and by stage and site of the tumor. However, there was no prior hypothesis to support any of these latter analyses, which were only performed to compare with previously published data. The frequencies of genotypes in cases and controls were compared using McNemar's test for matched case-control studies which provides greater power than an unmatched study (26). In addition, heterogeneity and contingency table analysis and χ^2 test for trend were used.

Data were analyzed using the program SPSS Release 6.0 (SPSS Inc., Chicago, IL) and EpiInfo Version 6.02 (Centers for Disease Control, Atlanta, GA; WHO, Geneva, Switzerland). ORs were calculated using the CIA software program (Confidence Interval Analysis Version 1.1; Martin Gardner and the BMJ, 1991).

Results

GSTP1. The overall frequency of *GSTP1* alleles and genotypes is shown in Table 1. The frequencies for the Val-105 allele were 0.33 for controls and 0.31 for cases. The frequencies for the Val-114 allele were 0.1 for controls and 0.08 for cases. No individuals were detected who were positive for Val-114 but not for Val-105.

In the controls, 11.7% of individuals were homozygous and 42.7% were heterozygous for Val-105, and 44.9% were homozygous for Ile-105. In the cases, the figures were 7.6, 45.2, and 46.9%, respectively. This compares to a homozygous rate for Val-105 of 6.5% in the normal Edinburgh population and 9.1% in Norway and heterozygous rates of 42.5 and 39.4%, respectively (15, 27). There was no difference between cases and individually matched controls in the frequency of homozygotes for Val-105 (OR = 0.57; 95% CI, 0.25–1.2) or the frequency of individuals who possessed at least one Val-105 allele (OR = 1.01; 95% CI, 0.65–1.6).

In the controls, 15.6% of individuals possessed the Val-114 variant (*GSTP1*C* allele), and in the cases, 14.3% of individuals (OR = 1.0; 95% CI, 0.5–1.9). There were six individuals homozygous for Val-114 (all but one in the control group), but this difference was not significant (OR = 0.18; 95% CI, 0.01–1.58).

GSTT1. For *GSTT1*, 19.8% of cases and 16.9% of controls were null. There was no difference in the frequency of the null genotype between matched cases and controls by McNemar's test (OR = 1.21; 95% CI, 0.63–2.0). Analysis by sex showed no difference between cases and their matched controls.

There was no significant difference between cases and controls for any of three age bands by McNemar's test, but for three age bands, the frequency of the *GSTT1* null genotype appeared to become less with increasing age. This is represented in Table 2. A χ^2 test for trend of the *GSTT1* null

Table 1 Allele and genotype frequencies of the *GSTP1* gene in cases and controls[a]

	Cases (n = 196)	Controls (n = 178)
Allele frequency		
*GSTP1*A*	278 (0.70)	238 (0.67)
*GSTP1*B*	91 (0.23)	83 (0.23)
*GSTP1*C*	28 (0.07)	35 (0.10)
Genotype frequency		
**A/*A*	92 (0.47)	82 (0.45)
**A/*B*	69 (0.35)	54 (0.31)
**A/*C*	20 (0.10)	20 (0.11)
**B/*B*	6 (0.035)	12 (0.07)
**B/*C*	8 (0.04)	5 (0.02)
**C/*C*	1 (0.005)	5 (0.03)

[a] The values given are actual numbers, with frequencies in parentheses. The genotype frequencies were in Hardy-Weinberg equilibrium for both the cases and controls.

Table 2 Frequency of *GSTT1* null genotype by age[a]

	Age, yr (no. of matched pairs)		
	<65 (n = 54)	65–75 (n = 73)	>75 (n = 50)
Cases	15/54 (27.7%)	14/73 (19%)	6/50 (12%)
Controls	12/54 (22%)	12/73 (16.4%)	6/50 (12%)

[a] The values given are actual numbers with percentage frequencies in parentheses. χ^2 test for trend gave P = 0.14 for the cases.

genotype frequency with age in either the cases or controls did not reach statistical significance (P = 0.143 for cases).

The *GSTT1* null genotype was found in 10 of 24 (41.7%) of the Dukes' A cases, 14 of 69 (20.2%) of the Dukes' B cases, 9 of 66 (13.6%) of the Dukes' C cases, and 4 of 15 (26.6%) of the Dukes' D cases. The *GSTT1* null genotype was more frequent in Dukes' A tumors than in more advanced tumors (OR = 3.36; 95% CI, 1.23–9.15).

GSTM1. The *GSTM1* null genotype was found in 52% of all cases and 50.8% of controls (OR = 1.04; 95% CI, 0.67–1.65). Analysis by subgroups of age, sex, and stage or site of the tumor showed no significant differences (data not shown).

As reported previously, 73 of 174 (42%) of the cases and 75 of 174 (42.5%) of the controls were fast acetylators (21).

Analysis of Combination Genotypes. Genotyping results were not available for all subjects and all assays, and if the matching of cases and controls had been maintained, considerable power would have been lost from the study. Therefore, all combination genotypes were examined using χ^2 and using all of the cases recruited. The combination of slow acetylator genotype with the *GSTT1* null genotype was found in 29 of 195 cases and 12 of 172 controls (OR = 2.33; 95% CI, 1.1–5.0). Heterogeneity analysis showed that the interaction between slow *NAT2* and *GSTT1* null was statistically significant, albeit at a marginal level (P = 0.049; Table 3).

There were no differences in the distribution of the *GSTP1* genotypes between cases or controls according to *GSTT1*, *GSTM1*, or *NAT2* genotypes.

Examination of the segregation of null and positive genotypes for *GSTT1* and *GSTM1* showed that they were randomly distributed in the cases. The occurrence of the putative "worst" combination of *GSTM1* null and *GSTT1* null was found in 8.8% of all cases and 6.6% of controls (not significant). There was also no difference in the frequency of the "best" combination genotype (positive for both *GSTT1* and *GSTM1*) between cases and controls (39% of cases and 36% of controls; not significant). In both the cases and the controls, the frequency of the *GSTM1* null genotype

Table 3 Heterogeneity analysis of the interaction between the combination of acetylator and *GSTT1* genotypes[a]

GSTT1 genotype	*NAT2* genotype	
	Fast	Slow
Wild-type	1	0.79 (0.49–1.26)
Null	0.41 (0.16–1.09)	2.24 (1.003–4.99)

[a] The values given are ORs between cases and controls, with 95% CIs in parentheses. The *GSTT1* wild-type/*NAT2* fast genotype was defined as odds ratio = 1, and odds ratios for other combinations were calculated relative to this.

was similar in both fast and slow acetylators, and there was no difference in the proportions of the combination genotypes between the two groups (data not shown).

Discussion

This population-based study suggests that variant alleles in the *GSTT1*, *GSTM1*, and *GSTP1* genes are unlikely to convey moderate increase in susceptibility to colorectal cancer, although the possibility of a small effect was not excluded. There has been conflicting evidence concerning the role of GST polymorphisms in susceptibility to colorectal cancer. Single studies have demonstrated an association between the *GSTT1* or *GSTM1* null genotype and colorectal cancer, although these have not been confirmed in further studies (10–14). The only study that has found a significant difference between cases and controls with respect to the *GSTM1* genotype was only marginally statistically significant (OR = 1.8; 95% CI 1.2–2.7) and was performed using cases recruited in Edinburgh and controls from three localities throughout the United Kingdom (10). The reasons for the differences between studies are not clear but could be related to methods of recruitment or type I statistical errors. Similar considerations could apply to the discrepancies seen in the studies of *GSTT1* null genotype. It seems unlikely that the *GSTM1* null genotype could predispose to colorectal cancer because *GSTM1* is only expressed at low levels in the colon (28). There appears to be no information available at present concerning *GSTT1* expression in human colon.

GSTP1 seems a more likely candidate susceptibility gene because it is expressed at high levels in the colon and because it has been demonstrated to play a role in heterocyclic amine deactivation (3, 28). In fact, in one previous publication on the Ile-105→Val polymorphism in relation to colorectal cancer, it appears that homozygous possession of the Ile-105 variant may actually protect from colorectal cancer, although the authors only examined for the effect of homozygous Val-105 (37 of 100 colorectal cancer cases and 79 of 155 controls homozygous for Ile-105; OR = 0.56 95% CI, 0.33–0.97; Ref. 15). It would be interesting to know the *in vitro* effect of the *GSTP1* polymorphisms on heterocyclic amine detoxification.

The population frequency of the Val-114 variant has been reported in two recent reports (15, 29). The frequency of both the *GSTP1* polymorphisms was remarkably similar in the present study to that reported in Australian and American Caucasians (34, 33, and 33% Val-105 in Australia, England, and America, respectively; and 7, 8, and 9% Val-114, respectively). We did not find any individuals with Val-114 but not Val-105, and we allocated genotype on the basis that the two polymorphisms are in strong linkage disequilibrium. However, it has recently been reported that there is a rare allele (*GSTP1*D*) that has the Val-114 polymorphism with Ile-105 (29). Our assay will have classified any subjects with the genotype *GSTP1*B/GSTP1*D* as *GSTP1*A/GSTP1*C*, but because the **B/*D* genotype is very rare (15, 29)

and the majority of **A/*C* genotype assignments will be correct, this is unlikely to have affected the overall outcome of the study.

Individuals with the combination slow acetylator-*GSTT1* null genotype were also more frequent in cases than controls. The relationship between acetylator status and colorectal cancer susceptibility is complicated, with some studies showing increased risk to individuals with the fast acetylator type and others showing no increased risk (18–23). In one study, fast acetylator individuals with high fried meat intake had an OR of 6.04 (95% CI, 1.34–55) of developing colorectal cancer, although fast acetylator status alone was not a risk factor (21). This suggests that slow acetylators must be at increased risk from other factors. This study suggests that this factor may be the *GSTT1* null genotype, and if this finding is confirmed in larger studies, it could help to explain some of the heterogeneity between previous studies of colorectal cancer and acetylator status.

Further analyses, which were performed for comparison with previous studies but for which there was no prior hypothesis, showed a number of interesting findings, although because of the small sample size these are best regarded as preliminary and need confirming in other studies. The *GSTT1* null genotype appeared to be less common with advancing age in both cases and controls, although this failed to reach statistical significance. This is particularly interesting because similar findings were noted in another study of colorectal cancer (11). It may be that the *GSTT1* null genotype gets less frequent with increasing age, although it is unlikely that a single genetic polymorphism could be responsible for such a significant increase in overall mortality. Because the finding was stronger in cases than controls, it is possible that individuals with the null genotype develop their tumors at a younger age than individuals that do express the enzyme. This finding needs to be investigated further in prospective studies. The *GSTT1* null genotype was also found more frequently in early rather than late tumors. This observation may have arisen by chance due to multiple analyses. However, if it were to be confirmed in other studies, it would suggest that the *GSTT1* null genotype is protective against progression of the tumor. Previous observations of an excess of the *GSTM1* null genotype in proximal cancers were not confirmed, suggesting that this may also have been a chance observation (10).

In general, this study has not confirmed previous suggestions of a role for GST polymorphisms in colorectal cancer susceptibility. However, secondary analyses have revealed some interesting associations that justify further investigation.

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