Polymorphisms in the glutathione *S*-transferase mu cluster are associated with tumour progression and patient outcome in colorectal cancer

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Abstract. Glutathione *S*-transferase (GST) enzymes catalyse the detoxification of by-products of reactive oxygen species and are thus important in cellular defence mechanisms. The GSTs are polymorphic with allelic variants encoding isoforms with functional differences. GST polymorphism has been associated with susceptibility and clinical outcome in patients with cancer. In this retrospective cohort, we have investigated associations between common *GSTM1, GSTM3* and *GSTP1* polymorphisms with factors known to influence clinical outcome and patient survival in colorectal cancer. Significant linkage disequilibrium was demonstrated between *GSTM1* and *GSTM3* alleles (P≤0.001). We identified no significant associations between the $GSTP1^{Ile105Val105}$ polymorphism and any clinical outcome parameters or patient survival. However significant associations were demonstrated with mu class GSTs. Those patients who were *GSTM1* null presented less frequently with poorly-differentiated tumours (P=0.038). Furthermore, patients who were *GSTM3 AA* were less likely to present with advanced stage tumours (T-stage, P=0.036 and Dukes' classifications, P=0.012) or distant metastases (P=0.017) when examined alone. Upon further examination of the effect of linkage disequilibrium, we found that, in *GSTM1* null individuals, *GSTM3 AA* (compared with other *GSTM3* genotypes combined) had longer disease-free survival (HR=0.54, 95% CI 0.30-0.98, P=0.044). Thus, the *GSTM3 AA* genotype is associated with improved prognosis especially in those with *GSTM1* null. Our findings suggest that the *GST* mu gene cluster mediates tumour characteristics and survival in patients with colorectal cancer.

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Introduction

Colorectal cancer is the third most common cancer worldwide and the World Health Organisation estimates that 945,000 new cases and 492,000 deaths occur yearly (1). The aetiology of colorectal cancer is believed to have both familial and environmental factors. It is estimated that familial colorectal cancer syndromes account for 5-15% of cancers (2), with the remainder of colorectal cancers comprising sporadic colorectal carcinoma, where interaction between genetic and environmental factors is proposed. Environmental factors including the contribution of diet-derived carcinogens (e.g. polycyclic aromatic hydrocarbons; PAH) have been proposed in some (3) but not all studies (4). Indeed, heterocyclic aromatic amines (HAAs) formed during cooking of meats have been shown to be colonic carcinogens in experimental animals (5). As glutathione *S*-transferase (GST) enzymes are involved in PAH metabolism, genetically-determined inter-individual differences in the host's ability to detoxify such carcinogens may be important and thus mediate susceptibility to malignancies associated with environmental PAH exposure (6).

Glutathione *S*-transferases (GSTs) are among the candidate genes implicated in many malignant neoplasms, including colorectal tumours (7). The GSTs, a supergene family of phase II detoxification enzymes, form a protective mechanism against chemical carcinogenesis (8). There are 8 classes of cytosolic GST and polymorphisms have been identified in several of these genes (9). Each class consists of several isoenzymes and have, in some cases, partially overlapping substrate selectivity (10).

In the case of *GSTP1*, three alleles have been identified apart from the wild-type *GSTP1*A*. *GSTP1*B* contains an A→G transition at nucleotide +313, *GSTP1*C* contains a C→T transition at nucleotide +341 in addition to the A→G transition, while *GSTP1*D* contains only the C→T transition at nucleotide +341. The single nucleotide substitution (A→G) at position +313 of the *GSTP1* gene results in replacing isoleucine with valine at amino acid 105. The $GSTP1^{Val105}$ variant, compared to $GSTP1^{Ile105}$, confers a 7-fold higher catalytic efficiency for PAH diol epoxides, but a 3-fold lower efficiency for 1-chloro-2, 4-dinitrobenzene (11,12). The C→T transition at nucleotide +341

causes an alanine to valine substitution at amino acid 114 and may augment the effect of the A>G +313 substitution (12). GSTP1 is widely expressed in normal human epithelial tissues and has been shown to be over-expressed in colon cancer (13). Drug-resistant tumours have also been found to contain increased levels of GSTP1 (14). GSTP1 directly participates in detoxification of platinum compounds and through transfection of antisense complementary DNA in colon cancer cell lines, GSTP1 was found to be an important mediator of both intrinsic and acquired resistance to platinum (15). Molecular epidemiology studies of GSTP1 associations with colorectal cancer are varied. Loktionov *et al* (16) and others (17) have found no associations between *GSTP1* and colorectal cancer risk. While Stoehlmacher *et al* (18) showed associations between $GSTP1^{Ile105}$ and reduced disease-free survival.

Of the five mu class GST on chromosome 1, *GSTM1* demonstrates a common gene deletion (*GSTM1*0* allele), leading to an absence of enzymatic activity (19), while *GSTM1*A* and *GSTM1*B* alleles encode monomers that form active enzymes (20). GST mu enzymes are more effective at detoxifying potential cytotoxic and genotoxic epoxides than other GSTs (21). GSTM1 has also been implicated in development of certain cancers (including colorectal cancers) and response to treatment (22). In addition, GSTM1 appears to be a modifier of GST alpha induction (23), the only GST that efficiently detoxifies N-acetoxy-PhIP (a representative of HAA carcinogens) (23). Further, GST remove isothiocyanates thought to be anti-carcinogenic. Thus, isothiocyanates will remain in the body for longer in those individuals who are *GSTM1* null (24). Higher *GSTM1* null frequencies have been observed in several case control studies in colorectal cancer (25,26). GSTM1 null has also been associated with an early age of onset in colorectal cancer (27). While other studies fail to confirm any association between *GSTM1* null and risk of colorectal cancer (17,28,29).

GSTM3 is biallelic with the alleles, *GSTM3*A* and *GSTM3*B*, differing by a 3-bp deletion in the latter that creates a motif for the YingYang1 (YY1) transcription factor. The 3-bp deletion in intron 6 of *GSTM3*B* also potentially leads to higher expression (30). Furthermore, *GSTM3*B* is in linkage disequilibrium with *GSTM1*A*. Loktionov *et al* (16) found associations between *GSTM3*B* frequency in patients with distal colorectal cancers particularly when combined with the *GSTM1* null genotype.

High risk GST genotypes (e.g. *GSTM1* null) have also been reported to be associated with somatic changes in tumour tissue such as p53 or K-ras mutations (31,32). If patients with low activity GST genotypes are more likely to have somatic changes generally associated with more aggressive tumour phenotypes (33), we could speculate that GST genotype may therefore be associated with survival in colorectal cancer patients.

While many studies have examined the association between GST genotypes and susceptibility to colorectal cancer (16,17, 25-29), few have examined outcome parameters (18). Of these latter studies, there are discrepancies in their findings. In this study, our aims were to: i) confirm (or otherwise) the previously identified associations between GST genotype and age at diagnosis, tumour location, degree of differentiation and Dukes' stage, ii) assess associations between genotype and additional clinical parameters (survival, tumour infiltration, host lymphocyte response) and iii) propose an explanation for the observed differences between studies on the role of GST in colorectal cancer.

Patients and methods

Patients. Northern European Caucasian patients with operative and histological confirmation of adenocarcinoma of the colon and rectum (n=443) were recruited from the University Hospital of North Staffordshire. Patients were recruited in a cross-sectional manner and followed-up for 2-15 years (mean age ± SD 67.1±10.8 years; 53.50% male). The patient group was selected randomly on the basis of their peripheral blood DNA availability. They do not represent any particular clinical subgroup and parameters were typical of colorectal cancer patients available at this centre. Only patients with sporadic colorectal cancer undergoing potentially curative surgery, at least locally (as in the case of metastatic disease), were included. Blood was obtained in the pre-operative period, to reduce possibility of contamination from any subsequent blood transfusion. All samples were collected with Local Research Ethics Committee approval and informed consent.

Clinical data. Clinical details, including operative and histological data, were obtained retrospectively from case notes and histology reports from a specialist colorectal histopathologist (Drs J. Elder, V. Smith). Clinical data were collected for established outcome markers: tumour site (right, left, rectum), tumour differentiation (well, moderate, poor), modified Dukes' stage, TNM classification (direct local invasion, number of positive lymph nodes, distant metastases; American Joint Committee on Staging Cancer) and survival. We also collected data on the less well recognised parameters of outcome: host lymphocyte reaction (HLR) and tumour margin morphology (pushing, invasive). The survival period was defined as the time interval from the date of operation to the date of death or the date last confirmed to be alive.

PCR. Genotyping was performed using DNA obtained from peripheral blood collected into EDTA. *GSTM1* genotypes were defined using a PCR assay that identifies *GSTM1* null homozygotes (*GSTM1*0/*0*), *GSTM1*A/*B* heterozygotes and the *GSTM1 A* (*GSTM1*A/*0* or *GSTM*A/*A*) and *B* (*GSTM1*B/*0* or *GSTM*B/*B*) phenotypes (34). The *GSTM3 AA*, *AB* and *BB* genotypes were identified by amplifying the exon 6/7 regions of *GSTM3* and differentiating *GSTM3*A* from *GSTM3*B* by digestions with *Mnl*I (35). The $GSTP1^{Ile105Ile105}$, $GSTP1^{Ile105Val105}$ and $GSTP1^{Val105Val105}$ genotypes were identified using primers to exon 5 (34). Data were available on 329 patients for GSTP1, 362 patients for GSTM1 and 303 patients for GSTM3.

Statistical analysis. All statistical analyses were performed using Stata, version 8.0 (Stata Corporation, TX). Chi-squared tests were used to test for homogeneity between groups (e.g. tumour location). Ordered logistic regression was used to assess increasing/decreasing genotype frequencies and interactions between genotypes in ordered categories (e.g. modified Dukes' stage). Since age at operation was not normally distributed,

Table I. The frequency of GST genotype according to clinical characteristics, in the total case group.

	All cases									
	GSTP1 *Ile105Ile105*	*GSTP1* *Ile105Val105*	*GSTP1* *Val105Val105*	*GSTM1* *null*	*GSTM1* **A/*A* **0/*A*	*GSTM1* **B/*B* **0/*B*	*GSTM1* **A/*B*	*GSTM3* *AA*	*GSTM3* *AB*	*GSTM3* *BB*
Sex										
Male	86 (47.0)	77 (42.1)	20 (11.0)	100 (52.6)	51 (26.8)	35 (18.4)	4 (2.1)	122 (71.4)	43 (25.2)	6 (3.5)
Female	59 (40.4)	64 (43.8)	23 (15.8)	89 (51.7)	44 (25.6)	30 (17.4)	9 (5.2)	96 (72.7)	34 (25.8)	2 (1.5)
Tumour site										
Right	47 (49.5)	39 (41.1)	9 (9.5)	52 (52.0)	22 (22.0)	22 (22.0)	4 (4.0)	61 (75.3)	19 (23.5)	1 (1.2)
Left	31 (38.8)	32 (40.0)	17 (21.3)	45 (50.6)	26 (29.2)	16 (18.0)	2 (2.3)	50 (68.5)	19 (26.0)	4 (5.5)
Rectum	64 (45.1)	64 (45.1)	14 (9.9)	88 (54.7)	40 (24.8)	26 (16.2)	7 (4.4)	100 (71.9)	37 (26.6)	2 (1.4)
Tumour differentiation										
Well	17 (48.6)	13 (37.1)	5 (14.3)	24 (64.9)	6 (16.2)	5 (13.5)	2 (5.4)	19 (61.3)	11 (35.5)	1 (3.2)
Moderate	89 (40.6)	103 (47.0)	27 (12.3)	133 (54.1)	62 (25.2)	43 (17.5)	8 (3.3)	147 (73.5)	50 (25.0)	3 (1.5)
Poor	31 (49.2)	22 (34.9)	10 (15.9)	29 (43.9)	22 (33.3)	13 (19.7)	2 (3.0)	45 (73.8)	12 (19.7)	4 (6.6)
TNM classification										
Direct local invasion										
T1	6 (40.0)	6 (40.0)	3 (20.0)	9 (64.3)	2 (14.3)	3 (21.4)	0 (0.0)	11 (78.6)	3 (21.4)	0 (0.0)
T2	16 (40.0)	18 (45.0)	6 (15.0)	25 (59.5)	8 (19.1)	9 (21.4)	0 (0.0)	30 (85.7)	5 (14.3)	0 (0.0)
T3	58 (43.0)	61 (45.2)	16 (11.9)	75 (54.0)	34 (24.5)	25 (18.0)	5 (3.6)	94 (72.9)	32 (24.8)	3 (2.3)
T4	63 (48.1)	52 (39.7)	16 (12.2)	75 (47.8)	49 (31.2)	26 (16.6)	7 (4.5)	78 (66.7)	34 (29.1)	5 (4.3)
Lymph nodes										
N0	76 (41.5)	82 (44.8)	25 (13.7)	104 (52.0)	52 (26.0)	37 (18.5)	7 (3.5)	118 (73.8)	39 (24.4)	3 (1.9)
N1	42 (47.2)	40 (44.9)	7 (7.9)	49 (51.0)	29 (30.2)	17 (17.7)	1 (1.0)	63 (72.4)	22 (25.3)	2 (2.3)
N2	18 (42.9)	16 (38.1)	8 (19.1)	25 (54.4)	11 (23.9)	8 (17.4)	2 (4.4)	26 (61.9)	14 (33.3)	2 (4.8)
Distant metastases										
M0	111 (41.6)	118 (44.2)	38 (14.2)	152 (52.2)	75 (25.8)	54 (18.6)	10 (3.4)	183 (75.0)	55 (22.5)	6 (2.5)
M1	34 (54.8)	23 (37.1)	5 (8.1)	37 (52.1)	20 (28.2)	11 (15.5)	3 (4.2)	35 (59.3)	22 (37.3)	2 (3.4)
Tumour margin										
Pushing	31 (36.9)	41 (48.8)	12 (14.3)	41 (51.3)	18 (22.5)	21 (26.3)	0 (0.0)	61 (77.2)	18 (22.8)	0 (0.0)
Infiltrating	50 (47.6)	43 (41.0)	12 (11.4)	57 (50.9)	35 (31.3)	17 (15.2)	3 (2.7)	64 (66.7)	30 (31.3)	2 (2.1)
Modified Dukes' stage										
A	10 (35.7)	12 (42.9)	6 (21.4)	21 (70.0)	4 (13.3)	5 (16.7)	0 (0.0)	21 (84.0)	4 (16.0)	0 (0.0)
B	61 (41.5)	66 (44.9)	20 (13.6)	80 (49.7)	44 (27.3)	29 (18.0)	8 (5.0)	95 (74.8)	29 (22.8)	3 (2.4)
C	50 (45.5)	46 (41.8)	14 (12.7)	63 (52.5)	34 (28.3)	22 (18.3)	1 (0.8)	76 (71.0)	28 (26.2)	3 (2.8)
D	21 (55.3)	14 (36.9)	3 (7.9)	22 (53.7)	10 (24.4)	7 (17.1)	2 (4.9)	20 (54.1)	15 (40.5)	2 (5.4)
Host lymphocyte reaction										
Yes	49 (42.6)	53 (46.1)	13 (11.3)	68 (51.5)	36 (27.3)	24 (18.2)	4 (3.0)	80 (74.8)	25 (23.4)	2 (1.9)
No	36 (44.4)	31 (38.3)	14 (17.3)	36 (50.7)	19 (26.8)	16 (22.5)	0 (0.0)	53 (69.7)	21 (27.6)	2 (2.6)

Figures quoted in parentheses are percentages.

Mann-Whitney U tests were used for comparisons of ages between groups. Survival analysis was performed using Cox's proportional hazard regression models and survival curves were constructed using the Kaplan-Meier method. Patients who died within 30 days of surgery were classified as peri-operative deaths and were excluded from survival analyses.

Results

Conformation of GST genotypes to Hardy-Weinberg equilibrium. The frequencies of *GSTP1, GSTM1* and *GSTM3* genotypes in colorectal cancer patients are shown in Table I. Allele frequencies in the total cohort and upon stratification

Table II. Linkage disequilibrium between *GSTM1* and *GSTM3* genotypes.

GSTM3 genotypes	*GSTM1* genotypes **0/*0*	**0/*A* and **A/*A*	**A/*B*	**0/*B* and **B/*B*
AA	124 (61.1)	32 (15.8)	42 (20.7)	5 (2.5)
AB	28 (38.9)	34 (47.2)	8 (11.1)	2 (2.8)
BB	1 (12.5)	7 (87.5)	0 (0.0)	0 (0.0)

$\chi^2_6 = 44.4560$, P≤0.001

Figures in parentheses are percentages.

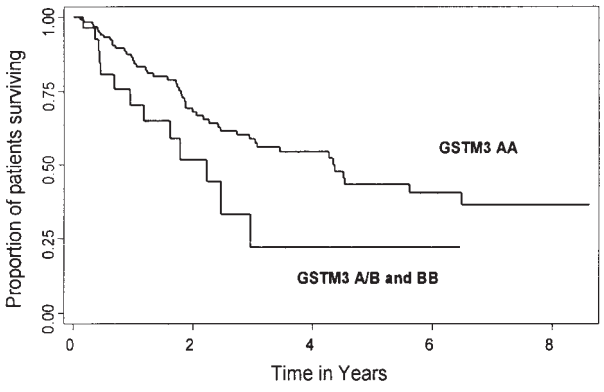

Figure 1. Kaplan-Meier survival curve for GSTM3. *GSTM3 AA* is associated with improved survival when compared to *GSTM3* heterozygotes and *GSTM3 BB* individuals.

by gender did not significantly depart from Hardy-Weinberg equilibrium. As expected, significant linkage disequlibrium was demonstrated between *GSTM1* and *GSTM3* alleles (P≤0.001). Thus 124 of 283 (61.1%) individuals who were *GSTM3 AA* were also *GSTM1* null compared with 1 of 8 (87.5%) of those with *GSTM3 BB* (Table II).

Associations between age, gender and GST polymorphisms. No significant differences were observed between *GSTP1, GSTM1* or *GSTM3* genotypes in male and female patients. Similarly, no significant differences were found between the three GST polymorphisms and the mean age at operation, though those patients with the *GSTM3 BB* genotype underwent operations 5.8 years later than those with other GSTM3 genotypes (data not shown).

Association of GST genotypes with parameters known to influence patient outcome. We next examined the data for association between *GSTP1* genotypes and factors that influence outcome in the total group. Within the colorectal cancer cases, we identified no significant association between *GSTP1* genotype and tumour differentiation, TNM classification and tumour margin morphology (Table I). Although not significant, we did observe that there was a trend for increasing $GSTP1^{Ile105Ile105}$ frequency, compared with $GSTP1^{Val105Val105}$ and $GSTP1^{Ile105Val105}$ combined, with more advanced Dukes' stage (ordered logistic regression; P=0.097) and that this genotype was more common in patients presenting with metastases (P=0.060, OR=1.71, 95% CI 0.98-2.98). We did observe that the distribution of GSTP1 genotypes was significantly different in tumours from different sites with more $GSTP1^{Val105Val105}$ patients presenting with left sided tumours (vs. right and rectum) compared to $GSTP1^{Ile105Ile105}$ and $GSTP1^{Ile105Val105}$ patients (P=0.007).

No significant associations were identified between *GSTM1* genotypes and tumour site (right, left and rectum) or tumour margin morphology (Table I), though the distribution of *GSTM1* genotypes in patients with a pushing tumour margin compared with infiltrating approached significance (P=0.097, χ^2_3=6.2). A significant trend was observed between *GSTM1* null frequency and tumour differentiation (ordered logistic regression; P=0.038).

No significant association between *GSTM3* genotype tumour differentiation, nodal involvement and tumour margin morphology were identified (Table I). However, a significant correlation was found between decreasing *GSTM3 AA* frequency (compared with *GSTM3 AB* and *BB* combined) with advancing tumour T-stage (ordered logistic regression; P=0.036) and increasing Dukes' stage (ordered logistic regression; P=0.012). A significant association was also observed between reduced *GSTM3 AA* frequency and presence of distant metastases (P=0.017, OR=0.48, 95% CI 0.47-0.88).

Association of GST genotypes with survival. We next investigated the relationship between *GST* genotypes and survival using Cox's proportional hazards regression. *GSTP1* did not affect overall survival ($GSTP1^{Ile105Ile105}$ reference category; $GSTP1^{Ile105Val105}$ HR=0.81, 95% CI 0.56-1.16, P=0.250, $GSTP1^{Val105Val105}$ HR=0.97, 95% CI 0.57-1.66, P=0.918). *GSTM1* null genotype was also not associated with survival, compared with other *GSTM1* genotypes (*GSTM1* null vs. rest; HR=1.04, 95% CI 0.759-1.44, P=0.789). However, *GSTM3* genotypes were non-significantly associated with overall survival (*GSTM3 AA* vs. rest; HR=0.70, 95% CI 0.48-1.02, P=0.065).

Analysis of the linkage disequilibrium between GSTM1 and GSTM3. Analysis was then performed to assess the impact of the linkage disequilibrium between *GSTM1* and *GSTM3*. Inclusion of *GSTM1* null genotype (vs. rest) in regression models showed that the decreasing frequency of the *GSTM3 AA* genotype remained significantly associated with increasing Dukes' stage (ordered logistical regression; P=0.009), advancing tumour stage (ordered logistical regression; P=0.020) and presence of metastases at operation (logistical regression; P=0.014). Furthermore significance was also observed as before between decreasing *GSTM1* null frequency and poorly-differentiated tumours (ordered logistical regression; P=0.024).

We next investigated the relationship between *GSTM1* and *GSTM3* and survival using Cox's proportional hazards regression. Inclusion of both variables in the model showed that the *GSTM3 AA* genotype was significantly associated with survival (HR=0.62, 95% CI 0.41-0.94, P=0.025) while the *GSTM1* null genotype was not (HR=1.43, 95% CI 0.96-2.14, P=0.081). *GSTM3 AA* was then found to be significantly associated with overall survival in *GSTM1* null individuals (corrected for age at operation and gender *GSTM3 AA*; HR=0.54, 95% CI 0.30-0.98, P=0.044) (Fig. 1), but not those

with other *GSTM1* genotypes (corrected for age at operation and gender, *GSTM3 AA*; HR=0.74, 95% CI 0.41-1.32, P=0.304). No significant interaction was found between the two loci, suggesting an additive, but largely independent effect of GSTM3 on survival.

Discussion

The majority of GST gene association studies in colorectal cancer to date have focussed on the impact of genotype on colorectal disease risk. Associations between GST genotypes and outcome have been reported in various diseases, with the data indicating different mechanisms for this effect. The present study was undertaken to assess the possible effects of gene polymorphisms within the GST family on factors that influence colorectal tumour characteristics and patient outcome. The subjects of the study represented a well-characterised ethnically homogeneous Caucasian population. Allele and genotype frequencies were similar to those reported elsewhere (34).

No associations were found between *GSTP1* genotypes and tumour characteristics. Although an association was observed between GSTP1 genotypes with left sided tumours, this is difficult to explain given the anatomy of the colon. Furthermore, no other associations with *GSTP1* genotype were observed suggesting that the observed association with tumour site should be treated with caution. We also found no associations between *GSTP1* genotypes and patient survival. However, Stoehlmacher *et al* (18) found that, of those colorectal patients who received 5-fluorouracil chemotherapy, those who also carried a *GSTP1 Val*^{105} variant allele had improved survival. Due to small patient numbers in our series on this therapy, we did not examine the influence of pre- and post-operative chemotherapy on patient survival.

We did find an association between *GSTM1* null and decreased frequency of poorly-differentiated tumours. However, given the lack of association with other outcome variables, this isolated finding would require further confirmatory data. To our knowledge, there is nothing in the literature to support this observation. We found no associations between *GSTM1* genotypes and patient survival. This is in agreement with Stoehlmacher *et al* (18) who also found no association in their metastatic colorectal cohort who had been treated with 5-fluorouracil chemotherapy. As *GSTM1* is expressed at low levels in colorectal tumour tissue (13), this may explain the lack of impact on survival.

While several studies have examined the effect of GSTM3 polymorphism on cancer susceptibility, few studies have looked for associations with clinical parameters or patient outcome. We observed that homozygosity for the *GSTM3***A* allele was less frequently associated with advanced tumours (T-stage and modified Dukes' stage) and distant metastases. Our findings also indicate that the *GSTM3 AA* genotype can be regarded as a protective factor against tumour progression and patient survival, especially in *GSTM1* null subjects. Our data suggest that this is due to additive, at least partially independent effects, of the two loci, rather than a synergistic interaction. We believe these results are more likely to be real due to the internal consistency of associations with *GSTM3***A*, though we recognise that none of the outcome parameters studied are independent. Stoehlmacher *et al* (18), however, found no association of *GSTM3* genotype with patient survival in their metastatic 5-fluorouracil treated colorectal cohort. The reasons for contradictory results between studies could be due to cohort heterogeneity or different patient treatments. The mechanism of the effect with *GSTM3* is unclear although the strong linkage disequilibrium between *GSTM3***B* and *GSTM1 A* may play a part (30). As suggested by Loktionov *et al* (16), it can be hypothesised that the absence of an additional recognition site for the YingYang1 (YY1) multi-functional transcription factor as observed in the *GSTM3***B* sequence is of importance. It is well documented that the YY1 transcription factor can both repress and activate transcription. However, molecular mechanisms controlling its behaviour are complex and are still not completely understood (36). The long GSTM1 deletion combined with the *GSTM3* alleles may result in structural changes to the DNA molecule within and around the GST mu region which may affect their expression. Observations of lower levels of GSTM3 expression in *GSTM1* null individuals (37) also suggest that interactions between GST mu genes are indeed important.

Although interesting, the numbers of individuals in some genotype groups were relatively low and these results should therefore be treated with caution. Furthermore, the data presented here has not been corrected for multiple testing. Using the Bonferroni procedure (to correct for multiple testing), all significance observed in this study would be lost. Though these results should be considered exploratory and need to be tested in an independent large confirmatory cohort, the internal consistency of the *GSTM3* results provides some element of confidence in the results.

This is the first study, however, to identify an association between *GSTM3* alleles and colorectal cancer tumour characteristics and outcome. We observed that linkage disequilibrium occurs between two closely associated members of GST mu on chromosome 1 (GSTM1 and GSTM3) and that *GSTM3 AA* is associated with less aggressive tumours and prolonged survival. We speculate that this association could be due to lower GSTM3 expression in tumours such as that observed in GSTM1 null individuals (37). Indeed lack of the YY1 motif in *GSTM3 AA* could potentially lead to lower expression in tumours (30). Further molecular epidemiological studies are needed to comprehensively investigate genotype interactions within the GST family in colorectal and other cancers.

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