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*IIA105Val105 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.

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 AG +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 translocation 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 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 a higher expression (30). Furthermore, 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 remain in the body for longer in those individuals who are null genotype. Lead ing to an absence of enzymatic activity (19), while GST mu enzymes are more effective at isothiocyanates (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 GSTM1*A'A') and B (GSTM1/B/ '0 or GSTM1'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 MbolI (35). The GSTP1Val105Ile, GSTP1Ile105Val and GSTP1Ile105Ile 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,
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 perioperative 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 are presented in Table I.
by gender did not significantly depart from Hardy-Weinberg equilibrium. As expected, significant linkage disequilibrium 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).

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

<table>
<thead>
<tr>
<th>GSTM3 genotypes</th>
<th>GSTM1 genotypes</th>
</tr>
</thead>
<tbody>
<tr>
<td>'0'/'0'</td>
<td>'0'/A and 'A'/A</td>
</tr>
<tr>
<td>AA</td>
<td>124 (61.1)</td>
</tr>
<tr>
<td>AB</td>
<td>28 (38.9)</td>
</tr>
<tr>
<td>BB</td>
<td>1 (12.5)</td>
</tr>
</tbody>
</table>

\[ \chi^2 = 44.4560, P \leq 0.001 \]

Figures in parentheses are percentages.

**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 GSTP 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*Val105Val105* frequency, compared with GSTP1*Ile105Val105* and GSTP1*Ile105Ile105* 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*Ile105Val105* 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, \( \chi^2 = 6.2 \)). 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*Ile105Ile105* 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, GSTM1 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 focused 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 Val105 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.

Acknowledgements

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References


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