Allelism at the glutathione S-transferase GSTM3 locus: interactions with GSTM1 and GSTT1 as risk factors for astrocytoma

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We describe studies to assess the influence of polymorphism in the human glutathione S-transferase GSTM3 gene on susceptibility to high grade astrocytoma. Immunohistochemical studies using a GSTM3-specific antiserum identified expression of the GSTM3 subunit in astrocytes. The relative levels of expression of GSTM1 and GSTM3 in brain cytosols were determined after resolution of these enzymes using chromatofocusing. We found no differences in the level of GSTM3 activity in individuals with GSTM1 null and those with GSTM1-positive genotypes (GSTM1*A, GSTM1*B and GSTM1*A/B). A case-control study was performed to determine if GSTM3 allelic polymorphisms influence susceptibility to high grade astrocytoma. After correction for differences in age and gender, GSTM3 AA was not significantly different in cases compared with controls. No significant interactions between GSTM3 AA and GSTM1 null were identified. The significant interaction between GSTM3 AA and GSTT1 null appeared to result from the strength of the main effect (GSTT1 null). The data show that while GSTM3 is expressed in astrocytes and contributes significantly to total GST activity in human brain, it does not appear to influence susceptibility to high grade astrocytoma. Further, unlike lung, there appears to be no relationship between the level of GSTM3 activity in brain and GSTM1 genotype.

Introduction

The glutathione S-transferase (GST*) GSTM1 locus has attracted much interest because homozygosity for the deleted GSTM1*0 allele is common and appears to confer altered susceptibility to various non-familial cancers (1-7). However, it is unclear why GSTM1 genotypes are significant in some cancers but not others, even though the gene is expressed in the appropriate target tissue. Thus, while GSTM1 is expressed in human brain (8-10) and studies in the rat show that the mu class Yb gene is predominantly expressed in astrocytes (11), GSTM1 null does not confer increased susceptibility to astrocytoma (12). In contrast, allelism at the theta class GSTT1 locus, a gene whose protein product shares some substrates with GSTM1, does influence susceptibility to this tumour, suggesting the importance of detoxification of unknown carcinogens (12). Recent studies suggest that the influence of GSTM1 on cancer risk may be mediated by polymorphism at other loci encoding detoxifying enzymes. Thus, data from human lung show that expression of the neighbouring mu class GSTM3 gene (13) is influenced by the GSTM1 genotype; GSTM1*0 homozygotes express less GSTM3 than subjects with other GSTM1 genotypes (4,14). This effect may result from allelism at GSTM3 (15). Two alleles, GSTM3*A and GSTM3*B, are identified. Importantly, GSTM3*B is in linkage disequilibrium with GSTM1*A and contains, in intron 6, a recognition motif for the YY1 transcription factor (15). These data suggest that the influence of GSTM1 on susceptibility is partly determined by interactions with GSTM3, a view supported by recent studies from this laboratory showing that individuals with the GSTM1 null/GSTM3 AA haplotype are at increased risk of multiple cutaneous basal cell carcinomas (16).

Although smoking is not a recognized risk factor for astrocytoma, the relevance of carcinogen metabolizing enzymes is shown by studies identifying various environmental xenobiotics, including nitroso compounds, methyl halides and polycyclic aromatic hydrocarbons, as causative factors (17,18) and the association of GSTT1 and cytochrome P450, CYP2D6 genotypes with risk of this tumour (12). Accordingly, we now describe studies to assess the role of GSTM3 in mediating susceptibility to astrocytoma. We describe, firstly, immunohistochemical studies to determine the site of GSTM3 expression in brain. Secondly, quantification of GSTM1 and GSTM3 isoenzymes in brain cytosols using chromatofocusing to assess whether GSTM1 genotype influences expression of GSTM3 and, thirdly, a case-control approach to compare the frequencies of GSTM3 genotypes in controls and patients with high grade astrocytoma and identify interactions with GSTM1 and GSTT1.

Materials and methods

Patient samples

Tissue and blood samples were obtained from unrelated Northern European Caucasians admitted to the North Staffordshire Hospital. Samples were collected with Ethical Committee approval and informed consent.

Immunohistochemical identification of GSTM3 expression in brain

Brain tissue from 16 patients suffering high grade astrocytoma was obtained from archive material in the Department of Histopathology. All tissues were fixed in 10% phosphate-buffered formalin and processed through graded alcohols and xylene to paraffin wax (19). Serial sections of 5 µm were cut, treated with hydrogen peroxide and covered with swine serum (diluted 1:3) (20). Sections were incubated (1 h, 20°C) with a rabbit polyclonal antiserum (diluted 1:100) specific for the GSTM3 subunit from human testis (16,20). A section not treated with primary antiserum served as the negative control. A positive control was provided by sections of human kidney. A biotinylated swine anti-rabbit secondary antibody (Amersham, UK) was used with an avidin-biotin-peroxidase complex (Dakopatts, Denmark). Peroxidase activity was developed with diaminobenidine tetrahydrochloride (Sigma, UK) (19). Astrocytes were identified using antiserum to glial fibrillary acidic protein in a procedure essentially as described for GSTM3 except an antigen retrieval step was used. A biotinylated rabbit anti-mouse serum was used as second antibody.
cells, as well as necrotic tissue, was weak. Staining of serial sections for glial fibrillary acidic protein showed the presence of many astrocytes in uninfiltrated tissue surrounding the tumour, but in all 16 samples, these cells demonstrated weak positivity for the GSTM3 subunit. However, at the edges of the uninfiltrated/tumour tissue interface all astrocytes demonstrated strong expression (Figure 1). No other brain cells demonstrated strong positivity for GSTM3.

Chromatofocusing studies
Figure 2 shows a representative chromatofocusing elution profile of brain cytosol from an individual with the GSTM1 A phenotype. Alpha class isoforms eluted first at pH 7.90-7.10 and pi class enzymes at pH 4.90-4.50. GSTM1 phenotypes of matched brains and livers, determined by their elution pH values (9), were similar in the 30 subjects studied and were confirmed by PCR analysis. Activity corresponding to the GSTM3-3 enzyme eluted at pH 6.00 and was identified in 28 of the 30 brain cytosols, but none of the matched liver samples. The mean contributions of GSTM3 and GSTM1 to total GST activity in the 30 brain cytosols studied were 14.7% (range 0-38.8%) and 6.1% (range 0-43.0%) respectively. The mean percentage contribution of GSTM3 to total GST activity in cytosols from individuals with GSTM1 null (15.3 ± 12.8%) was similar to those with GSTM1 A, GSTM1 B and GSTM1 A/B (14.0 ± 11.3%), as were mean specific activities (21.3 ± 18.4 and 28.0 ± 18.1 μmol/min/mg protein respectively).

Case–control study
Table I shows GSTM3, GSTM1 and GSTT1 genotype frequencies in controls and high grade astrocytoma cases. GSTM3 and GSTM1 frequency distributions and individual genotype frequencies in these groups were not significantly different. As expected (12), the frequency of GSTT1 null was significantly increased in the astrocytoma cases compared with controls ($P = 0.0063, \chi^2 = 7.48$). We used logistic regression to correct for age and gender differences between cases and controls in our assessments of GST genotypes. This showed that the frequency of GSTM3 AA in the astrocytoma cases was not significantly different to that in controls. Logistic regression with a step-up selection routine was used to determine which of the variables (gender, age, GSTM3, GSTM1

Results

Immunohistochemical identification of GSTM3
All brain cells, including astrocytes identified in tumour and perilesional, uninfiltrated tissue, demonstrated positivity for the GSTM3 subunit, though generally expression in these cells, as well as necrotic tissue, was weak. Staining of serial sections for glial fibrillary acidic protein showed the presence...
GSTM3 and susceptibility to astrocytoma

Fig. 2. Chromatofocusing profile of brain cytosol. The profile of a brain cytosol from a chromatofocusing column shows the elution of alpha class GST (GSTA1, GSTA2), GSTM1, GSTM3 and pi class enzymes. GST activity was determined using CDNB and reduced glutathione.

Table I. GSTM3, GSTM1 and GSTT1 genotype frequencies in controls and patients with high grade astrocytoma

<table>
<thead>
<tr>
<th></th>
<th>GSTM3 AA</th>
<th>GSTM3 AB</th>
<th>GSTM3 BB</th>
<th>GSTM1 null</th>
<th>GSTM1 A</th>
<th>GSTM1 B</th>
<th>GSTM1 A, B</th>
<th>GSTT1 null</th>
<th>GSTT1 A</th>
</tr>
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<tr>
<td>Astrocytoma (n = 89)</td>
<td>65 (73.0%)</td>
<td>22 (24.7%)</td>
<td>2 (2.2%)</td>
<td>51 (57.3%)</td>
<td>21 (23.6%)</td>
<td>13 (14.6%)</td>
<td>4 (4.5%)</td>
<td>30 (33.7%)</td>
<td>59 (66.3%)</td>
</tr>
<tr>
<td>Controls (n = 300)</td>
<td>221 (73.7%)</td>
<td>64 (21.3%)</td>
<td>15 (5.0%)</td>
<td>121 (57.3%)</td>
<td>56 (26.5%)</td>
<td>25 (11.8%)</td>
<td>9 (4.3%)</td>
<td></td>
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</tr>
<tr>
<td>Controls (n = 284)</td>
<td>121 (57.3%)</td>
<td>56 (26.5%)</td>
<td>25 (11.8%)</td>
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and GSTT1) best discriminated between cases and controls. In astrocytoma cases age, gender and GSTT1 null were selected. Interactions between GSTM3 AA and other genotypes were also studied using logistic regression in the presence of age and gender to determine whether the interactive terms were more significant than the main effects. No significant interactions with GSTM1 null were found and while the interaction between GSTM3 AA and GSTT1 null was significant ($P = 0.023$), it appeared to result from the strength of the GSTT1 null effect. Thus, when this interaction was assessed in the presence of the main effects (GSTT1 null alone and GSTM3 AA alone), the major contribution to risk (lowest $P$ value) was from GSTT1 null alone, rather than the interaction with GSTM3 AA.

Discussion

We have assessed the influence of the recently described polymorphism in GSTM3 on susceptibility to high grade astrocytoma. The two GSTM3 alleles identified differ by the YY1 recognition motif 5'-AAGATA-3', present in GSTM3*B, but not GSTM3*A (15). While the motif occurs in intron 6, intronic and extragenic sequences are important in mediating transcriptional activity at other enzyme loci (20), suggesting that GSTM3*A and GSTM3*B are differently regulated and have distinct effects on cancer susceptibility. We used an immunohistochemical approach to show expression of the GSTM3 subunit in astrocytes, and chromatofocusing to determine the relative contributions of GSTM1 and GSTM3 to total GST activity in brain tissue. These studies showed that GSTM3 makes a significant contribution to mu class GST activity in human brain that is similar to that of GSTM1. Accordingly, we carried out a case-control study to determine if this gene influences susceptibility to high grade astrocytoma alone or through interactions with GSTM1 or GSTT1.

While the factors that influence susceptibility to astrocytoma are unclear, the effectiveness of detoxification of blood borne chemicals may be important, as astrocytes provide a protective barrier between neurones and blood. This view is supported by studies showing that a variety of environmental pollutants, including polycyclic aromatic hydrocarbons and nitroso compounds, can induce central nervous system tumours (17,18) and by data showing that polymorphism at both GSTT1 and CYP2D6 influences risk of high grade astrocytoma (12). It is unclear why allelism at GSTT1 but not GSTM1 is influential, though data showing GSTM3 is the predominant mu class enzyme in human brain cytosol suggest that expression of GSTM3 may compensate for homozygous deficiency of GSTM1. This possibility is complicated by the identification of allelism at GSTM3 (15).

While several studies have shown expression of GSTM3 in cytosols prepared from brain homogenates, the cellular site of expression in human brain was unclear. This study has presented evidence that the gene is expressed in astrocytes, though generally expression was weak, with only some cells
at the boundary of the tumour demonstrating strong positivity. The reason for this phenomenon is unknown. Furthermore, the chromato-focusing studies show that GSTM3 contributes substantially to total GST activity in brain cytosols. Indeed, the contribution of the gene is likely to be greater than indicated by our results as the specific activity of GSTM3 towards CDNB (15.2 μmol/min/mg protein) is markedly lower than that of GSTM1 (~170 μmol/min/mg protein) (20). It is also noteworthy that, unlike lung, expression of GSTM3 appeared to be independent of the GSTM1 genotype. These data indicate different interactions between these two genes in lung and brain, possibly resulting from differences in YY1 expression.

While GSTM3 is expressed in astrocytes, our data show allelism at GSTM3 does not influence susceptibility to astrocytoma. Furthermore, interactive effects with GSTM1 or GSTT1 genotypes were not identified. These data contrast with studies in basal cell carcinoma showing that individuals with GSTM1 null/GSTM3 AA and GSTM3 AA/skin type 1 are at increased risk of multiple primary basal cell carcinoma (16). The reason for the apparent difference in significance of the GSTM3 polymorphism in these two cancers is unclear, particularly as the gene is expressed in both target tissues. They may reflect different causative substrates in the two pathologies and/or tissue-specific differences in YY1 expression.

The role of polymorphism in other putative candidates, such as that recently described in GSTP1 (21), as well as the GSTM3 polymorphism in these two cancers is unclear, with GSTM1 null/GSTM3 genotypes and smoking and asbestos exposure.

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References