Polymorphism at the Glutathione S-Transferase Locus GSTM3: Interactions with Cytochrome P450 and Glutathione S-Transferase Genotypes as Risk Factors for Multiple Cutaneous Basal Cell Carcinoma

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Abstract

The influence of polymorphism in the glutathione S-transferase, GSTM3 gene on susceptibility to cutaneous basal cell carcinoma (BCC) has been investigated. We have reported previously two GSTM3 alleles, GSTM3*A and GSTM3*B, distinguished by a recognition motif for the YY1 transcription factor in GSTM3*B. In this study, immunohistochemistry was used to identify GSTM3 expression in the epidermis of skin samples from 11 controls and 9 patients with BCC. A PCR method was used to identify GSTM3*A and GSTM3*B and thereby the GSTM3 AA, GSTM3 AB, and GSTM3 BB genotypes in 300 controls and 286 Caucasians with 1–35 primary BCCs. Genotypes at GSTM1, GSTT1, and the cytochrome P450 CYP1A1 and CYP2D6 loci were also determined. Frequencies of GSTM3, GSTM1, GSTT1, CYP2D6, and CYPIA1 genotypes in the cases and controls were not different. Dividing the BCC cases into groups of 92 patients with 1 lesion and 194 patients with 2–35 lesions showed that the frequencies of GSTM3 BB (2.6%) and GSTM1 A/B (1.3%) in the group with 2–35 tumors were almost significantly lower than in the group with 1 lesion (7.6%, exact P = 0.0061, x2 = 3.398; 6.5%, exact P = 0.055, x2 = 4.946, respectively). Within the cases with 2–35 tumors, a Poisson regression model was used to identify genotypes, characteristics such as skin type, and interactions between genotypes and characteristics associated with increasing numbers of tumors. This showed, after correction for male gender and age, that GSTM3 AA was not associated with risk of increased numbers of tumors, although in combination with skin type 1, GSTM1 null, and CYPIA1 M1M1, the genotype did confer increased risk (P < 0.001, rate ratio, 2.058; P < 0.001, rate ratio, 1.606; P < 0.001, rate ratio, 1.470, respectively). The data suggest that, like other allelic GST, GSTM3 influences cancer risk. As GSTM3 AA was associated with increased tumor numbers, it appears YY1 acts as an activator of the recognition motif in GSTM3*B.

Introduction

Polymorphism at the GST, GSTM1 locus has attracted much interest, because homozygosity for GSTM1*0 confers altered risk for several cancers (1–3). However, although GSTM1 null may be a susceptibility marker, data from some studies, including in lung cancer, are conflicting (1, 2). The reason for these discrepancies is unclear but may reflect the influence on detoxification of interactions between GSTM1 and other allelic loci encoding detoxifying enzymes. Thus, a model based only on the hypothesis that GSTM1 null confers increased risk may be too simplistic (1). Furthermore, there is evidence GSTM1*A and GSTM1*B have different effects on susceptibility to some pathologies, including bladder cancer and Crohn’s disease (1), and multiple cutaneous BCCs, in which GSTM1 A/B, but not GSTM1 A or GSTM1 B, is protective (4). The mechanism for these effects is unclear as the products of GSTM1*A and GSTM1*B demonstrate similar in vitro catalytic properties (3, 5). GSTM1 is one of five μ genes (M1–M5; Refs. 3 and 6) the products of which demonstrate overlap in their substrate specificities (3, 5), implying that the protein products of one μ-class locus will compensate functionally for the absence of other family members. Indeed, studies in the lung suggest coordinated expression of some μ-class genes; thus, GSTM1*0 homozygotes express less GSTM3 than do subjects with other GSTM1 genotypes (7). The mechanism for this observation is unknown but may be related to the finding that GSTM3 is also polymorphic, with two alleles, GSTM3*A and GSTM3*B, identified (8). Importantly, GSTM3*B is in linkage disequilibrium with GSTM1*A and contains in intron 6 a recognition motif for the YY1 transcription factor, which regulates gene expression from intragenic sites (8). The widely expressed YY1 factor influences the expression of many genes (9), suggesting that GSTM1*A and GSTM3*B are expressed at different levels and GSTM3 genotypes will confer different efficiencies in the metabolism of carcinogens. We propose, therefore, that assessment of the contribution of GSTM1 genotypes as susceptibility markers for cancer risk needs to take account of interactions with GSTM3. The nonfamilial skin cancers offer an interesting opportunity to test the hypothesis that allelism at GSTM3 influences risk, inasmuch as we have recently shown that GSTM1 null in combination with skin type 1 is associated with increased susceptibility to multiple BCCs (4, 10). Allelism at GSTT1 and the cytochrome P450 (CYP) CYP2D6 and CYPIA1 loci also influences the pathogenesis of multiple lesions (10, 11). Accordingly, we describe, first, immunohistochemical identification of GSTM3 in skin and, second, a case-control approach to compare GSTM3 genotype frequencies in controls and patients with BCC. Third, the case group was divided into patients with 1 tumor and those with 2–35 lesions. The patients with multiple lesions can be considered to have an increased susceptibility to BCC, and a cross-sectional study was performed to determine the influence of GSTM3 on susceptibility to multiple BCC and identify interactions with CYP and GST genotypes and characteristics such as gender and skin type.

Materials and Methods

Patient Samples. Genotypes were determined in leukocyte DNA from 286 English Caucasians attending the North Staffordshire and Royal Cornwall Hospitals suffering BCC [1–35 primary tumors per patient; median number of
tumors, 2; mean age, 68.3 ± 12.2 (SD) years; 58.4% males] during the period 1993–1995. These cases were divided into two groups: first, 92 patients who suffered one BCC (mean follow-up, 1.8 years; range, 8 months–11.5 years). These cases constituted a random sample of patients with one lesion from the participating centers from whom DNA was available. The second group comprised 194 patients with 2–35 tumors (mean follow-up, 7.5 years; range, 1.1–28.9 years). It was composed of 179 patients with 2–10 tumors and 15 patients with 11–35 tumors and represented about 60% of the cases in the two participating hospitals; the remaining patients were lost to follow-up. All patients were examined by a trained dermatologist (J. T. L., A. H. H., A. S., or B. B.) to obtain data on eye color (blue, green, or brown) and skin type (types 1–6; Ref. 10) and sex. Tumor numbers and the presence of other malignancies. Recurrences were excluded from the number of BCCs, as were cases with basal cell nevus syndrome or BCC and other cancers. Diagnoses were confirmed histologically.

Caucasian controls (300) from North Staffordshire and Cornwall [mean age, 62.0 ± 17.7 (SD) years; 41.1% males] without evidence of any malignancy were also recruited. These in- and outpatients suffered varicose veins, hernias, hemorrhoids, mild iron deficiency, mild hyperlipidemia, benign ovarian cysts, tension headaches, benign skin papillomas, benign breast lumps, and cerebrovascular accidents. Patient samples were collected with the approval of the Ethical Committees of the North Staffordshire and Royal Cornwall Hospitals and informed consent.

Immunohistochemical Studies. Normal skin from 11 patients was provided at operation for breast reduction. Skin from 9 patients suffering BCC was obtained from archive material in the Department of Histopathology, North Staffordshire Hospital, Stoke-on-Trent. All tissues were fixed in 10% phosphate-buffered formalin and processed through graded alcohols and xylene to paraffin wax (12). Serial sections (5 μm) were cut, treated with hydrogen peroxide, and covered with swine serum (diluted 1:3; Ref. 12). Sections were incubated (1 h, 20°C) with a rabbit polyclonal antiserum specific to GSTM3 (diluted 1:100; Refs. 3 and 5). The immunogen was purified from human testis (5). A section not treated with primary antiserum served as negative control. A positive control was provided by sections of human kidney. A biotinylated swine antirabbit secondary antibody (Amersham, United Kingdom) was used with an avidin-biotin-peroxidase complex (Dakopatts, Denmark). Peroxidase activity was developed with diaminobenzidine tetrahydrochloride substrate (Sigma Chemical Co., Poole, United Kingdom), counterstained with hematoxylin, dehydrated, mounted, and assessed (12).

Identification of Genotypes. GSTM3 genotypes were identified using primers to exon 6/7 (8). GSTM3*B was differentiated from GSTM3*A, which is 3 bp larger, by digestion with Mnll. DNA from GSTM3*A homozygotes, containing the additional Mnll site, gave fragments of 11, 51, 86, and 125 bp, whereas GSTM3*B homozygotes gave fragments of 11, 125, and 134 bp. GSTM1 genotypes were identified using ARMS-based PCR with primers to intron 6/exon 7 (4). GSTT1 null and expressing subjects were also identified using PCR (10). Two mutant CYP2D6 alleles (G→A transition at intron 6/exon 4 and bp deletion in exon 5) were identified (2, 10). Together, these assays are 90% predictive of phenotype in British Caucasians (2). The 3'-flanking region mutation (m1/m1 wild-type homozygotes) and the exon 7 Ile-Val mutation (Ile/Ile wild-type homozygotes) in CYP1A1 were detected using PCR with restriction digestion (11, 13).

Statistical Analysis. χ2 tests were used to examine for homogeneity between cases and controls. As some genotype frequencies were small, the StatXact-Turbo statistical package was used. The influence of genotypes and characteristics alone and in combination, was studied by logistic regression.

Fig. 1. Immunohistochemical identification of GSTM3 expression in skin samples from controls and patients with BCC. a, skin sample from control showing GSTM3 throughout the epidermis; b, sample from control showing variable GSTM3 expression in skin fold; c, skin sample from a control demonstrating weak expression of GSTM3 in the stratum spinosum (×100); d, GSTM3 expression in tumor cells in a BCC (×400).
Analysis of the effect of genotypes on BCC numbers was studied by applying a Poisson regression model (EGRET statistical package; SERC, 1993) to count data in cases with 2–35 tumors. In the model, the Poisson rate parameter (mean number of BCCs) is expressed as a function of a set of covariates (i.e., age, gender, and genotypes). The data were transformed to take account of all counts >2. This analysis was exploratory and not intended to be predictive, our objective being to identify covariates associated with the difference in transformed counts. A rate ratio, defined as the multiplicative effect of a change of a covariate by 1 was calculated (usually a change from 0 to 1). Thus, the rate ratio for males, 1, against females, 0, is mean number BCC in males/mean number BCC in females when gender alone is considered. This will change in the presence of other covariates.

Results

Immunohistochemical Identification of GSTM3. The GSTM3 subunit was found in all 11 skin samples from controls, although the extent of positivity varied markedly within sections and between subjects (Fig. 1, a–c). Generally, the intensity of staining increased from the basal layer toward the stratum granulosum (Fig. 1a), although in some subjects or other regions of the same section, the basal layer demonstrated no immunoreactivity (Fig. 1, b and c). Thus, in one control, positivity was greatest in the basal layer in one part of the section, with other parts showing increasing positivity from the basal layer to the stratum granulosum. In a further control, only a few cells of the inner stratum spinosum were weakly positive (Fig. 1c). In all samples, sebaceous glands, sweat glands, and arrector pili muscles gave strongly positive results. GSTM3 expression was also found in the tumors from nine BCC patients, although in six cases, only a few cells were positive (Fig. 1d). In eight cases, perilesional skin was available; three samples demonstrated increasing GSTM3 positivity from the basal layer to the stratum granulosum; and in five subjects, expression was confined to parts of the stratum spinosum.

Influence of GST Genotypes on Susceptibility. Table 1 shows GSTM3 genotype frequencies in controls and cases. Frequency distributions and genotype frequencies in these groups were not different. Similarly, frequency distributions and genotype frequencies for GSTM1, GSTT1, CYP2D6, and CYP1A1 were not significantly different from those published for North Staffordshire BCC cases and controls (10, 11). The proportion of males and the mean age of the BCC group were significantly greater than in controls (P < 0.001 in both cases). Logistic regression showed that the age- and gender-corrected proportions of GSTM3 AA in the cases and controls were not different. Similarly, the age- and gender-corrected proportions of GSTM3 AA in combination with GSTMI A, GSTMI null, GSTT1 null, CYP2D6 EM, or CYP1A1 mimi in cases and controls were not significantly different.

To determine whether GSTM3 genotypes were associated with risk of multiple tumors, the BCC cases were divided into patients with 1 lesion and those with 2–35 tumors (Table 1). The frequencies of GSTM3 BB and GSTM1 A/B in the group with 2–35 tumors were almost significantly lower than in the group with 1 lesion (exact P = 0.0601; $\chi^2 = 3.390$; exact P = 0.055; $\chi^2 = 4.946$, respectively).

Within the cases with 2–35 tumors only, Poisson regression was used to identify genotypes, individual characteristics and interactions between genotypes, and characteristics associated with increasing numbers of primary tumors. Thus, male gender and increased age were associated with a significant increase in the number of BCCs (P < 0.001). Neither GSTM3 AA nor GSTM1 A, GSTT1 null, or CYP1A1 Ile/Ile, alone or after correction for age and gender, were significantly associated with an increased number of BCC. However, after correction for age and gender, skin type 1, CYP1A1 mimi, CYP2D6 EM, and GSTM1 null were significantly associated with increased numbers of primary tumors. Thus, male gender and increased age were associated with a significant increase in the number of BCC.

Table 2 Age- and gender-corrected factors and interactions influencing number of BCCs

<table>
<thead>
<tr>
<th>Individual factors</th>
<th>P</th>
<th>Rate ratio</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Skin type 1 (n = 141)</td>
<td>&lt;0.001</td>
<td>2.019</td>
<td>1.616–2.523</td>
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<tr>
<td>CYP1A1 Ile/Ile, (n = 156)</td>
<td>&lt;0.001</td>
<td>1.978</td>
<td>1.424–2.747</td>
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<tr>
<td>CYP2D6 EM (n = 146)</td>
<td>&lt;0.001</td>
<td>1.608</td>
<td>1.246–2.074</td>
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<tr>
<td>GSTMI null (n = 143)</td>
<td>0.002</td>
<td>1.436</td>
<td>1.144–1.802</td>
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</table>

<table>
<thead>
<tr>
<th>Interactions between factors</th>
<th>P</th>
<th>Rate ratio</th>
<th>95% CI</th>
</tr>
</thead>
<tbody>
<tr>
<td>GSTM3 AA, skin type 1 (n = 141)</td>
<td>&lt;0.001</td>
<td>2.058</td>
<td>1.628–2.601</td>
</tr>
<tr>
<td>GSTM3 AA, GSTT1 null (n = 143)</td>
<td>&lt;0.001</td>
<td>1.606</td>
<td>1.287–2.004</td>
</tr>
<tr>
<td>GSTM3 AA, CYP1A1 mimi (n = 156)</td>
<td>&lt;0.001</td>
<td>1.470</td>
<td>1.184–1.827</td>
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Discussion

We have studied the influence of the newly described polymorphism at GSTM3 on risk of multiple BCC. This is the first report of allelic diversity in a $\mu$-class gene other than GSTM1 as a candidate for susceptibility. Our immunohistochemical experiments showed specific expression of the gene in skin from cases and controls. The influence of GSTM3 on susceptibility was studied, first, by comparing genotype frequencies in controls with those in the BCC cases. Second, the frequency of genotypes in patients with 1 tumor was compared with that in patients with 2–35 lesions, a group that appears at increased risk of BCC. Third, a Poisson regression model was used in the cases with 2–35 tumors to identify genotypes and characteristics that influence tumor numbers.

Patients with a BCC are at high risk of suffering further lesions (14, 15). Importantly, this risk depends on the number of tumors present. Thus, in subjects with 1 lesion, the 5-year risk is 27%, and in those with 10 or more tumors, the risk is 90% (14, 15), indicating that accrual is not just dependent on time but rather that some subjects
have an increased susceptibility (15). Risk increases with age, male gender, exposure to UV, and skin types associated with burning without tanning (skin type I; Ref. 15). Exposure to UV constitutes an oxidative stress, and it is likely that individual differences in response to this stress will mediate risk. μ-class GSTs are attractive candidates for susceptibility to skin cancers, including BCC, as recent studies from our laboratory suggest that GSTM1 contributes to detoxification of the products of UV-induced oxidative stress. For example, GSTM1 null is associated with anti-Ro (but not anti-La) antibodies in patients with systemic lupus erythematosus (16). Significantly, production of anti-Ro antibodies in the absence of anti-La is associated with photosensitivity. Furthermore, GSTM1 null in combination with skin type 1 confers increased susceptibility to multiple BCCs (10, 11). If the influence of GSTM1 results from its metabolism of products of UV-induced oxidative stress, GSTM3 is also a candidate, as it utilizes hydrogen peroxide and cumene hydroperoxide (3). Our finding that GSTM3 is expressed in the epidermis and basal layer is compatible with the view that this enzyme is part of local antioxidant defenses. Thus, the basal layer includes the stem cells believed to be the targets for UV-induced damage from which BCCs arise.

Our analysis did not identify differences in the frequencies of GSTM3 genotypes in the total BCC group and controls or between cases with 1 tumor and those with 2–35 lesions, although it is noteworthy that the difference in the frequency of GSTM3 BB in these two case groups approached significance, suggesting that this relatively common genotype is protective and worthy of further study. Within the cases with 2–35 tumors, a Poisson regression model was used to study the association between genotypes and number of lesions. This analysis showed that GSTM3 AA alone was not associated with an increased number of tumors. However, combinations of GSTM3 AA with skin type 1, GSTM1 null, or CYP1A1 m1m1 were significantly associated with increased tumor numbers. Indeed, the rate ratio for the interaction between GSTM3 AA and skin type 1 (2.058) was the highest identified. The classification of skin type 1 defines extreme sensitivity to UV that results in an inflammatory response but no tanning. Presumably, individuals with this skin type and GSTM3 AA or GSTM1 null are less able to metabolize the products of the oxidative stress associated with UV exposure. The finding of a significant interaction between GSTM3 AA and GSTM1 null complements studies showing lower levels of immunohistochemical positivity for GSTM3 in the lungs of subjects with GSTM1 null (7) and suggests a similar effect in skin. These data may reflect the linkage of GSTM3*B with GSTM1*A (8) and the consequent association of GSTM3*A with the other GSTM1 alleles, the majority of which will be GSTM1*. Our finding of a significant interaction between CYP1A1 m1m1 and GSTM3 AA provides further support for the view that polymorphism in CYP genes influences susceptibility to multiple BCC (10). Thus, CYP1A1 is expressed in skin and is induced by UV exposure (17). The consequences of the 3′-downstream mutation are unclear but may confer increased inducibility, suggesting that CYP1A1 m1m1 provides less effective detoxification on exposure to carcinogens (17, 18). Our finding of an interaction between CYP1A1 and a μ-class GST is compatible with data showing that GSTM1 null is associated with high inducibility of CYP1A1 transcription (19). The mechanism for the increased susceptibility to BCC conferred by CYP2D6 EM is also unclear, although recent studies indicate the importance of this gene in skin and brain tumors (10, 11, 20).

Our data suggest that GSTM3 AA confers increased risk of multiple BCC. Although the mechanism is unclear, we speculate that the YY1 transcription factor acts as a GSTM3 inducer in skin via the 5′-AAAGATA-3′ motif in GSTM3*B. YY1 expression is altered by many molecules, including growth factors (9), suggesting that GSTM3*B homozygotes are better able to induce expression of the gene after UV exposure. Our results provide further evidence for the importance of effective metabolism of potential carcinogens in skin and support the view that other factors in addition to UV exposure are determinants of disease risk.

References

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