Polymorphisms at the glutathione S-transferase GSTM1, GSTT1 and GSTP1 loci: risk of ovarian cancer by histological subtype

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The phase II glutathione S-transferases (GSTs) GSTT1, GSTM1 and GSTP1 catalyse glutathione-mediated reduction of exogenous and endogenous electrophiles. These GSTs have broad and overlapping substrate specificities and it has been hypothesized that allelic variants associated with less effective detoxification of potential carcinogens may confer an increased susceptibility to cancer. To assess the role of GST gene variants in ovarian cancer development, we screened 285 epithelial ovarian cancer cases and 299 unaffected controls for the GSTT1 deletion (null) variant, the GSTM1 deletion (null) variant and the GSTP1 codon 104 A→G Ile→Val amino acid substitution variant. The frequencies of the GSTT1, GSTM1 and GSTP1 polymorphic variants did not vary with tumour behaviour (low malignant potential or invasive) or p53 immunohistochemical status. There was a suggestion that ovarian cancers of the endometrioid or clear cell histological subtype had a higher frequency of the GSTT1 and GSTM1 deletion genotype than other histological subgroups. The GSTT1, GSTM1 and GSTP1 genotype distributions did not differ significantly between unaffected controls and ovarian cancer cases (overall or invasive cancers only). However, the GSTM1 null genotype was associated with increased risk of endometrioid/clear cell invasive cancer [age-adjusted OR (95% CI) = 2.04 (1.01–4.09), \( P = 0.05 \)], suggesting that deletion of GSTM1 may increase the risk of ovarian cancer of these histological subtypes specifically. This marginally significant finding will require verification by independent studies.

Introduction

Ovarian cancer is the main cause of death among women with gynaecological malignancies. The lifetime risk of developing ovarian cancer is 1 in 99 in Australia (1), no different from the incidence world wide (2). While mutations in the breast cancer susceptibility genes BRCA1 and BRCA2 or in the mismatch repair genes hMSH2 and hMLH1 are responsible for most ‘hereditary’ ovarian cancers from multiple case families (3), the majority of Australian patients with ovarian cancer present without such a strong family history of cancer. It is likely that ‘low risk’ genes may account for at least some predisposition to these apparently ‘sporadic’ ovarian cancers, since risk of mortality due to any cancer is increased in relatives of ovarian cancer patients (4).

Candidate low risk ovarian cancer predisposition genes include those that are involved in carcinogen metabolism and contain common functional allelic variants that affect gene expression or protein function. These include the phase II glutathione S-transferases (GSTs) glutathione S-transferase θ1 (GSTT1), glutathione S-transferase μ1 (GSTM1) and glutathione S-transferase π1 (GSTP1), which catalyse the glutathione-mediated reduction of exogenous and endogenous electrophiles with broad and overlapping substrate specificity. It has been hypothesized that GST functional variants associated with less effective detoxification of potential carcinogens may confer an increased susceptibility to cancer, especially in the presence of environmental stresses such as smoking or exposure to UV radiation (5). Deletion variants or null alleles exist for the GSTT1 and GSTM1 genes and these present biochemically as a failure to express protein (6–8). These alleles are common, with the GSTT1 and GSTM1 null genotypes occurring in ~10–20% and 40–65%, respectively, of the Caucasian population (9). The GSTP1 gene contains several polymorphisms (10–12). Functional significance has been demonstrated for an exon 5 A→G transition resulting in a codon 104 Ile→Val amino acid substitution, with altered heat stability and specific activity of the Val-containing isof orm (11,13). These and other GST variants have been investigated as risk factors for the predisposition to numerous cancers, including lung, bladder, breast, colorectal, gastric, liver, larynx and skin cancer, with conflicting results between and within cancer types (9,14–16).

There are few reported studies investigating the effects of GST polymorphisms on the risk of ovarian cancer and, due to small sample sizes, these have been limited in their power to detect modest changes in risk. No association between the GSTT1 and GSTM1 null variants and ovarian cancer was observed in three separate studies of 103 patients and 115 controls (17), 84 cases and 325 controls (18) and 146 cases and 80 controls (19). However, immunohistochemical analysis of p53 expression in 63 ovarian tumours included in the study of Sarhanis et al. (18) suggested that p53 overexpression was associated with the GSTM1 null and possibly also GSTT1 null genotype, leading these authors to conclude that failure to detoxify products of oxidative stress may result in genetic damage. In addition, GSTT1 and GSTM1 null genotypes were shown to be associated with poorer survival and a reduced progression-free interval in 148 epithelial ovarian cancer cases (20). Although there are no reported ovarian cancer case–control studies of the GSTP1 codon 104 Ile→Val variant, high concentrations of GSTP1 protein in human ovarian follicular fluid suggest that this enzyme plays an important role in

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folllicular detoxification processes (21), while a small study of GSTP1 expression in ovarian tumours suggested that GSTP1 levels were lower in higher grade tumours and in serous malignant tumours (22).

We have undertaken a large case–control comparison to assess the role of the GSTTI, GSTM1 and GSTP1 genetic variants as risk factors for epithelial ovarian cancer and to investigate the association between these variants and ovarian tumour pathology, including expression of p53.

Materials and methods

Subjects
Details of cases and controls have been described previously (23). Briefly, incident cases of epithelial ovarian adenocarcinoma or cystadenoma were ascertained from the Royal Brisbane Hospital, Queensland, Australia from 1985 to 1996. Only cases with low malignant potential (LMP) (n = 44) or invasive (n = 241) tumours were included in the analyses presented here. LMP tumours have some but not all the features of malignancy. LMP tumours were characterized by cellular proliferation with stratification and pleomorphism but no stromal invasion, whereas invasive tumours were characterized by invasion of the ovarian stroma (24). This subseries comprised 183 serous, 29 mucinous, 32 endometrioid, 15 clear cell carcinoma, eight mixed Mullerian, six mixed and four undifferentiated tumours, as well as eight of unknown histology. For analysis, the mixed Mullerian and mixed histologies were treated as a single mixed group. Age at diagnosis ranged from 21 to 95 years, with an average age of 59.9 years.

Controls (n = 299) were adult female unrelated monozygotic twins (only one per pair) from a sample of 3348 twins of almost exclusively European descent, recruited through the volunteer National Australian Twin Registry for the Semi-Structured Assessment of the Genetics of Alcoholism research study (25). Control subjects were selected to match as closely as possible the date of birth distribution observed for ovarian cancer patients, namely one-third from each of 1900–1925, 1926–1938 and 1939–1970. Age at interview ranged from 30 to 90 years, with an average age of 50.9 years.

Ethical clearance for collection of information and blood from cases and controls was given by the Queensland Institute of Medical Research Ethics Committee.

Genotype detection

Germline DNA was prepared from whole blood as described previously (23). The homozygous GSTTI and GSTM1 gene deletion polymorphisms were screened by multiplex PCR amplification, using primer sets lying within the GSTTI and GSTM1 genes to identify the presence of these gene sequences. An unrelated primer set homologous to the 5'-untranslated region of the estrogen receptor gene generating a larger control amplicon was included to verify successful amplification of each DNA sample. Primer sequences and product sizes are detailed in Table I. The 10 μl reaction mix contained 30 ng DNA, primers (400 nM each), deoxyribonucleotide triphosphates (200 nM), 1X Perkin Elmer Taq polymerase buffer, 1 U Taq polymerase and 1.5 mM MgCl2. Amplifications were incubated for 5 min at 94°C, four cycles of 94°C for 20 s, 65°C for 20 s and 72°C for 20 s, four cycles of 94°C for 20 s, 65°C for 20 s and 72°C for 20 s and 34 cycles of 94°C for 20 s, 65°C for 20 s and 72°C for 20 s, followed by a 10 min extension at 72°C. Reaction products were resolved on 4.5% Nusieve gels, with positive and negative control samples included in all PCR and gel runs to detect possible contamination problems and gel loading and typing inconsistencies.

The GSTP1 exon 5 Ille105→Val A→G polymorphism (26) was detected using the Perkin Elmer ABI Prism 7700 Sequence Detection System (SDS) for multicentre real time or end-point forensic PCR detection (Perkin Elmer Applied Biosystems, Foster City, CA; catalogue no. 7700-01-220/240). A 92 bp PCR product was amplified using the forward and reverse primers as detailed in Table I. Ban1 enzyme digestion and high resolution agarose gel electrophoresis was used to identify AA and GG homozygote DNA controls required as standards for the SDS allelic discrimination assay. Using the standard protocol for SDS allelic discrimination assay, fluorescently labelled probes 5'-6-carboxyfluorescein (FAM)-CG CCT GCA AAT ACA TCT CCC TCA TCTA-6-carboxylfluorescein (TAMRA)-3' and 5'-6-carboxy-4,7,7'-tetramethylrhodamine (TET)-CGC TGC AAG TAC GTC TCC CTC ACG TAC TAC-TAMRA-3' were used to detect the A and G alleles, respectively. The final concentration of reagents in the PCR mix (reaction volume 25 μl) was 1X TaqMan Universal PCR Master Mix (Perkin Elmer catalogue no. 4304437), 50 nM forward primer, 360 nM reverse primer, 200 nM FAM-A probe and 75 nM TET-G probe. Reaction mix was added to 30 ng genomic sample DNA that had been pre-dried in 96-well plates. PCR reactions were incubated in the ABI 7700 SDS PCR machine for 2 min at 50°C and 10 min at 95°C, followed by 45 two-step cycles of 15 s at 95°C and 1 min at 64°C. Genotype analysis was performed on amplified samples using the ABI PRISM 7700 software, following standard procedures. Repeatability of the ABI PRISM 7700 SDS genotyping was assessed by re-analysis of a subsample of 122 DNA samples, selected on the basis of DNA availability. Successful re-amplification of samples generated confirmatory genotype results in all instances.

p53 immunohistochemical analysis

Immunohistochemistry was performed as detailed by Webb et al. (27) and tumours were classified p53-positive if >10% of cells stained positive for p53. Tumour tissue suitable for immunohistochemical analysis was available from only 83 of the ovarian cancer cases included in this study, 69 of which were invasive tumours.

Statistical analysis

The Student’s t-test was used to compare the large difference of cases and controls. The Hardy–Weinberg equilibrium (HWE) assumption was assessed for case and control groups by comparing the observed numbers of different genotypes with those expected under HWE for the estimated allele frequency and comparing the Pearson goodness-of-fit statistic with a χ2 distribution on 1 degree of freedom. HWE was tested for the GSTTI locus only, since the GSTTI and GSTM1 genotyping methods did not distinguish between null/ non-null heterozygotes and non-null/non-null homozygotes. Differences in the distribution of GSTTI, GSTM1 and GSTP1 genotype amongst cases stratified according to tumour behaviour (LMP versus invasive), histology and p53 status were assessed by the χ2 test. Logistic regression was used to assess the association between genotype and age at interview or parity amongst controls and age at onset amongst cases. Logistic regression with adjustment for age was used to calculate odds ratios (ORs) and 95% confidence intervals (CIs) for the association between GST genotype and ovarian cancer status. SPSS v.9.0 and Ottutil software were used for statistical analyses.

Results

GSTTI, GSTM1 and GSTP1 genotype data were successfully generated for the majority of cases (285, 285 and 282 subjects, respectively) and controls (295, 297 and 292 subjects, respectively). p53 immunohistochemical analysis was carried out on 14 LMP and 69 invasive tumours from the ovarian cancer cases included in this study. Ovarian cancer cases were stratified by tumour behaviour (LMP or invasive), histology and p53 status to investigate the possibility of GST genotype heterogeneity between subgroups (Tables II and III). There is evidence to suggest that the somatic mutational pathway may differ between invasive and LMP ovarian tumours (28) and invasive tumours are more likely than LMP to exhibit p53 overexpression (29). Thus, only invasive ovarian cancers (comprising the largest subgroup for stratification by tumour behaviour) were included in the histological subtype and p53
analyses shown in Tables II and III. There were too few LMP tumours to consider similar stratification of this group.

There was no significant difference between the GSTTI and GSTMI null genotype frequencies (Table II) of LMP and invasive cancers. Although differences with respect to histology within invasive ovarian cancers were not statistically significant overall, the frequency of the GSTTI null genotype was significantly greater ($P = 0.04$) in endometrioid and clear cell subtypes (29 and 33%, respectively) than in the serous subtype (17%). Endometrioid and clear cell carcinoma are considered to be histologically and epidemiologically similar (30–32) and the serous subgroup was chosen for comparison since it is the single largest histological subtype. The GSTMI genotype showed similar but statistically significant heterogeneity across histological subtypes ($P = 0.02$), also with higher frequencies of the GSTMI null genotype in endometrioid and clear cell cancers (77 and 67%, respectively) compared with serous cancers (47%) ($P = 0.002$). There was little difference in GSTPI genotype distribution between the subgroups for tumour behaviour or histology (Table III).

The results in Tables II and III do not support published evidence that p53 positivity is associated with GSTTI or GSTMI gene deletion, as was reported by Sarhanis et al. (18). Similarly, there was no association between p53 status and GSTPI genotype status. Results were unchanged when the data were re-analyzed using the p53 scoring method of Sarhanis et al. (18), which deemed sections to be positive if 50% of nuclei in tumour cells demonstrated strong expression ($P = 0.5, 0.4$ and $0.6$ for GSTTI, GSTMI and GSTPI, respectively). There was also no association between p53 positivity and GSTTI, GSTMI or GSTPI genotype status among the small sample of LMP tumours tested for p53 expression ($P = 1.0, 0.4$ and $0.04$, respectively).

Case–control comparisons were performed separately for cases overall and for invasive cases only (Table IV). Although age did not appear to be associated with genotype of any of the three GST systems ($P > 0.2$ for all ovarian cancer cases, invasive cases only and unaffected controls), ORs were adjusted for age since cases were significantly older than controls (mean 59.9 versus 50.9 years, $P < 0.001$). Epidemiological information such as parity was not available for cases and it was thus not possible to calculate ORs with adjustment for this or other known ovarian cancer risk factors. However, there was no association between GSTTI genotype and parity among controls ($P = 0.4, 0.2$ and $0.04$ for GSTTI, GSTMI and GSTPI, respectively). There was no evidence for deviation from HWE for the GSTTI locus in cases ($P > 0.7$ for total sample, LMP and invasive tumour forms) or controls ($P = 0.8$).

There was little difference in the frequencies of the putative high risk GST genotypes in controls compared with cases (either overall or invasive only), with none of the ORs being significantly different from unity (Table IV). Possession of both GSTTI and GSTMI null genotypes also did not affect risk of ovarian cancer either overall or for invasive cancer only (Table IV). Similarly, possession of all three putative high risk genotypes, GSTTI null, GSTMI null and GSTPI GG/AG, was not associated with disease [age-adjusted OR (95% CI) = 0.81 (0.35–1.85) and 0.91 (0.39–2.14)] for all cancers and invasive cancers, respectively.

Our data suggest that the endometrioid and clear cell cancers had a greater frequency of GSTTI and GSTMI null genotypes (Table II) than the other histological subtypes. These histologically similar and epidemiologically related subtypes (30–32) were pooled for comparison with the control group. The pooled case group included the cases presented in Tables II–III and an additional two invasive ovarian cancer cases with mixed endometrioid/clear cell histology. After adjustment for age there was no difference in GSTPI genotype frequency between the endometrioid/clear cell cases and unaffected controls [OR (95% CI) = 0.93 (0.32–2.74) for the GG genotype versus AA reference]. However, both the GSTTI and GSTMI null genotypes were more common in invasive endometrioid/clear cell cases (31 and 73%, respectively) than in controls (19 and 55%, respectively). The age-adjusted OR (95% CI) associated with invasive endometrioid/clear cell tumours was 1.78 (0.88–3.60) for GSTTI null ($P = 0.1$) and 2.04 (1.01–4.09) for GSTMI null ($P = 0.05$). For analysis of the combined GSTTI+GSTMI genotype using GSTTI+GSTMI non-null as the reference group, the age-adjusted OR (95% CI) was 2.09.
GSTM1 deletion genotypes were more common in both endo-ethnicity is also unlikely, since controls were almost exclusively GSTT1 GST GSTM1 deletion polymorphism or There was no signifi-histological subgroups were also compared with controls. logy may have different aetiologies (33 Discussion more common in endometriosis patients than in controlshistological subgroups or clear cell subtype (30,31,36,37), the data are also in

Table IV. Association of GST genotype status with ovarian cancer

<table>
<thead>
<tr>
<th>Genotype</th>
<th>Controls</th>
<th>Cases, overall</th>
<th>Cases, invasive only</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>n (%)</td>
<td>n (%) OR [95% CI]</td>
<td>n (%) OR [95% CI]</td>
</tr>
<tr>
<td><strong>GSTT1</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-null</td>
<td>239 (81)</td>
<td>228 (80) 1.00 [reference]</td>
<td>192 (80) 1.00 [reference]</td>
</tr>
<tr>
<td>Null</td>
<td>56 (19)</td>
<td>57 (20) 1.05 [0.68–1.61]</td>
<td>49 (20) 1.02 [0.64–1.62]</td>
</tr>
<tr>
<td><strong>GSTM1</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Non-null</td>
<td>135 (45)</td>
<td>126 (44) 1.00 [reference]</td>
<td>111 (46) 1.00 [reference]</td>
</tr>
<tr>
<td>Null</td>
<td>162 (55)</td>
<td>159 (56) 1.03 [0.73–1.45]</td>
<td>130 (54) 0.90 [0.90–1.31]</td>
</tr>
<tr>
<td><strong>GSTP1</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>AA</td>
<td>114 (39)</td>
<td>121 (43) 1.00 [reference]</td>
<td>101 (42) 1.00 [reference]</td>
</tr>
<tr>
<td>AG</td>
<td>135 (46)</td>
<td>130 (46) 0.92 [0.64–1.34]</td>
<td>110 (46) 0.98 [0.65–1.45]</td>
</tr>
<tr>
<td>GG</td>
<td>43 (15)</td>
<td>31 (11) 0.75 [0.43–1.31]</td>
<td>27 (11) 0.85 [0.47–1.53]</td>
</tr>
<tr>
<td>AG/GG</td>
<td>178 (61)</td>
<td>161 (56) 0.89 [0.62–1.26]</td>
<td>137 (58) 1.02 [0.64–1.62]</td>
</tr>
<tr>
<td><strong>GSTT1/GSTM1</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Both non-null</td>
<td>106 (36)</td>
<td>101 (35) 1.00 [reference]</td>
<td>91 (38) 1.00 [reference]</td>
</tr>
<tr>
<td>GSTT1 null, GSTM1 non-null</td>
<td>27 (9)</td>
<td>25 (9) 0.95 [0.50–1.80]</td>
<td>20 (8) 0.78 [0.39–1.57]</td>
</tr>
<tr>
<td>GSTM1 null, GSTT1 non-null</td>
<td>131 (45)</td>
<td>127 (45) 1.00 [0.68–1.46]</td>
<td>101 (42) 0.83 [0.55–1.25]</td>
</tr>
<tr>
<td>Both null</td>
<td>29 (10)</td>
<td>32 (11) 1.12 [0.62–2.05]</td>
<td>29 (12) 1.05 [0.56–1.97]</td>
</tr>
</tbody>
</table>

aOR adjusted for age.

(0.61–7.19) for GSTT1 null (P = 0.2), 2.24 (0.95–5.27) for GSTM1 null (P = 0.07) and 3.90 (1.36–11.14) for both GSTT1 and GSTM1 null (P = 0.01).

To further investigate the histological heterogeneity of GSTM1 genotype frequency shown in Table II, the other histological subgroups were also compared with controls. There was no significant difference ($\chi^2$ test) in the frequency of GSTM1 null genotype frequency for cases with serous (P = 0.7), mucinous (P = 0.3) or mixed (P = 0.8) histology and the age-adjusted ORs for all of these histological subgroups were not significantly different from unity.

**Discussion**

The data presented in this study of 285 ovarian cancer cases and 298 unaffected controls provide no evidence that the genotypes defined by the GSTT1 deletion polymorphism, GSTM1 deletion polymorphism or GSTP1 A→G codon 104 polymorphism influence the overall risk of ovarian cancer. This study had 80% power at a significance level of 5% to detect an overall 1.6- to 1.7-fold increased risk of ovarian cancer in individuals with a putative high risk GST genotype. It is unlikely that real associations between GSTT1, GSTM1 or GSTP1 genotype and ovarian cancer risk could have been masked by differences in parity between cases and controls, since there was no association between genotype distribution and parity among controls. Confounding due to differences in ethnicity is also unlikely, since controls were almost exclusively of European descent (25) and the same would most likely be true for the cases, given that ovarian cancer is rare in other population groups (2). We acknowledge that this control group is not a true population-based sample, but believe that these twin controls, drawn from a variety of educational and socio-economic backgrounds and sampled in cities across Australia (25), are unlikely to be systematically genetically different from the general Australian population.

There was, however, a suggestion that the GSTT1 and GSTM1 deletion genotypes were more common in both endometrioid and clear cell ovarian cancer histological subtypes. Although the invasive endometrioid/clear cell subgroup was too small (n = 48) for reliable analysis, comparison with the control group suggested that deletion of the GSTM1 gene and, to a lesser extent, the GSTT1 gene, might be involved in the aetiology of endometrioid/clear cell ovarian cancer. This is consistent with prior evidence that tumours of different histology may have different aetiologies (33–35) and, specifically, that endometrioid and clear cell carcinomas are aetologically related (30–32,36,37). Given that endometriosis is considered a possible risk factor for ovarian cancer of the endometrioid or clear cell subtype (30,31,36,37), the data are also in accordance with the finding from a small French study that deletion of GSTM1 and, possibly, also deletion of GSTT1 is more common in endometriosis patients than in controls (38,39). It is also of interest that an increased frequency of the GSTM1 null genotype was observed in a sample of 80 endometrial cancer patients compared with 60 controls [64 versus 47%, OR (95% CI) = 2.0 (0.9–4.2), P = 0.06 (40), since endometrioid and clear cell ovarian tumours are histologically similar to adenocarcinoma of the endometrium (41) and ovarian cancer and endometrial cancer share epidemiological risk factors such as low parity (42,43), obesity (44) and oestrogen replacement therapy (32).

Analysis of p53 expression was possible for a subset of the ovarian cancer samples included in this study. The data from 69 invasive ovarian cancer cases included in this study do not support the hypothesis of Sarhanis et al. (18) that overexpression of p53 is associated with the GSTM1 and, possibly, GSTT1 null genotypes. Our study had 97% power at a significance level of 5% to detect a 2-fold difference in GSTM1 null genotype frequency between p53 negative and positive cases, as reported by Sarhanis et al. (18). The study by Sarhanis et al. (18) was of similar size with respect to p53 immunohistochemical analysis (n = 63), but the characteristics of the total ovarian cancer sample differed with respect to histology from the invasive sample presented here. Our study sample included more serous (64 versus 43%) and fewer endometrioid (11 versus 17%), mucinous (10 versus 14%) and undifferentiated (1 versus 19%) tumours. It is possible that the overall higher frequency of endometrioid and mucinous tumours in the study
of Sarhanis et al. (18) may explain the difference in findings between the two studies, since the frequency of GSTT1/GSTM1 gene deletions in these ovarian cancer histological subtypes appears to be greater compared with other subtypes (Table II). However, in our study there was no significant difference between p53 positive and negative samples with respect to the proportion of GSTT1 or GSTM1 null genotypes for either endometrioid/cler cell carcinoma or serous tumours (data not shown).

In conclusion, the GSTP1 codon 104 Val variant does not appear to be associated with ovarian tumour behaviour (LMP versus invasive), histology or p53 immunohistochemical status or altered ovarian cancer risk. There is also no convincing evidence to suggest that the GSTT1 or GSTM1 null genotype influences ovarian tumour behaviour or p53 immunohistochemical status or is associated with ovarian cancer risk overall. However, there is some evidence to suggest that these null genotypes may specifically increase the risk of endometrioid and clear cell carcinoma histological subtypes. We recognize that this observation is based on a small subset of the ovarian cancer sample studied and thus needs to be verified by replication in independent studies. It would also be of great value if such studies were designed to investigate possible interactions between the GSTT1 and GSTM1 null genotypes and exogenous or endogenous carcinogen exposure, since it is most likely that these detoxification genes may modify the risk of cancer induced by chemical carcinogens.

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