Low $O^6$-alkylguanine DNA-alkyltransferase activity in normal colorectal tissue is associated with colorectal tumours containing a GC→AT transition in the K-ras oncogene

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$O^6$-alkylguanine DNA-alkyltransferase (ATase) provides protection against the toxic, mutagenic and carcinogenic effects of alkylating agents, principally by removing the pro-mutagenic lesion $O^6$-alkylguanine from DNA. Differences in ATase activity in human tissue may thus determine mutational susceptibility. As GC→AT transitions, which can be induced by $O^6$-alkylguanine in DNA, are commonly observed in the K-ras oncogene of alkylating agent induced animal tumours and in human colorectal tumours, we have examined whether differences in ATase activity may affect the risk of K-ras mutations in humans with colorectal tumours. ATase activity in normal tissue from individuals with a K-ras mutation in colorectal tissue and more specifically a GC→AT transition (but not a transversion mutation) was significantly lower than that in individuals without a mutation ($P < 0.01$). Thus, individuals with low ATase activity in normal tissue (i.e. below the median) were at increased risk of having a transition (OR 10.1; 95% CI 1.9–99.0), but not a transversion mutation (OR 1.7; 95% CI 0.3–12.2). There were no significant differences in ATase activity in tumour tissue, although codon 12 and 13 -ras mutations in humans with colorectal tumours were found in 23 of 52 patients undergoing surgery with tumours of the rectum ($n = 25$), caecum ($n = 12$), sigmoid colon ($n = 21$), transverse colon ($n = 2$) or hepatic flexure ($n = 1$) and stored at −70°C.

Materials and methods

Tissue samples
Tumour and macroscopically normal tissue was obtained from 61 patients undergoing surgery with tumours of the rectum ($n = 25$), caecum ($n = 12$), sigmoid colon ($n = 21$), transverse colon ($n = 2$) or hepatic flexure ($n = 1$) and stored at −70°C.

K-ras mutation analysis
DNA was extracted from normal and tumour tissue using phenol-based methods, and codon 12 and 13 K-ras mutations were detected using a PCR based restriction endonuclease-site mutation assay followed by DNA sequencing (8).

Assay of ATase activity
Extracts of normal and tumour tissue were prepared by sonication and ATase activity measured in cell-free extracts by monitoring the transfer of radioactive methyl groups from substrate DNA to protein; activity was expressed in relation to the protein content of the tissue extract under excess substrate conditions.

Analysis of results
ATase activity was not normally distributed so the Mann–Whitney U-test was used to compare the levels of activity between the different groups. For descriptive purposes, however, we have also summarized the results as mean ± SD in Table I. Differences in mutational frequencies were investigated using the chi-square and Fishers exact tests.

Results

ATase activity and K-ras mutations in colorectal tissue
ATase activity was detected in all samples with levels ranging from 52-342 and 35-451 fmol/mg protein in normal and tumour tissue, respectively (Figure I). Inter-individual variation in ATase activity was thus 6.6- and 12.9-fold in normal and tumour tissue, respectively. K-ras mutations were found in 23 individuals: six mutations were found in macroscopically normal tissue (8). The frequency of mutations in tumour tissue was significantly higher than that found in normal tissue ($\chi^2 = 5.36, P < 0.05$).
Fig. 1. ATase activity in (A) normal and (B) tumour tissue from individuals with colorectal tumours stratified on the basis of the presence (mut+) or absence (mut-) of a K-ras mutation and the presence of a GC→AT transition (G-A) and of a GC→TA or GC→CG transversion (G→C/T) in the mutated K-ras oncogene. Results from individual patients are presented (open symbols) together with a box-plot showing the median and the 10–90% percentiles of ATase activity. *Difference in ATase activity significantly different from that in mut-group using the Mann-Whitney U-test (P<0.01).

Table I. K-ras mutational status and ATase activity in colorectal normal and tumour tissue

<table>
<thead>
<tr>
<th>Mutation</th>
<th>ATase activity (fmol/mg protein)</th>
<th>Normal tissue (SD)</th>
<th>Tumour tissue (SD)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No mutation</td>
<td>137 ± 67 (39)</td>
<td>173 ± 111 (35)</td>
<td></td>
</tr>
<tr>
<td>G→A transition</td>
<td>92 ± 29 (15)</td>
<td>109 ± 37 (13)</td>
<td></td>
</tr>
<tr>
<td>G→C/T transversions</td>
<td>108 ± 57 (7)</td>
<td>151 ± 89 (7)</td>
<td></td>
</tr>
<tr>
<td>All mutations</td>
<td>97 ± 39 (22)</td>
<td>124 ± 62 (20)</td>
<td></td>
</tr>
</tbody>
</table>

a Detected in both macroscopically normal and tumour tissue.

Mutation
d Insufficient sample from one individual (with a mutation) for ATase assay.

Individuals with low ATase activity in normal tissue (i.e. below the median) were at increased risk of having a GC→AT transition (OR 11.4; 95% CI 1.2–522.6) in their colorectal tissue (Table II).

As we could not exclude the possibility that a K-ras mutation might, in some way, reduce ATase expression in mutated cells, we then excluded from the analysis those individuals in whom mutations were found in macroscopically normal tissue. When this was done, there were still significantly more GC→AT transitions in individuals whose ATase activity was below the median (112 fmol/mg protein) than in individuals whose ATase activity was above the median (Table II): 27% (8/27) versus 4% (1/28), respectively (Fishers exact test, P = 0.01). Individuals with low ATase activity in normal tissue (i.e. below the median) were at increased risk of having a GC→AT transition in their tumour tissue (OR 11.4; 95% CI 1.2–522.6; Table II).

Relationship between ATase activity in tumour tissue and K-ras mutations

As the association between ATase activity in normal tissue and K-ras mutations may have been the result of the disease process itself, we examined the relationship between ATase activity in tumour tissue and K-ras mutations (Figure 1B).

Though ATase activity in tumour tissue from individuals was lower in individuals with a mutation or with a GC→AT transition, this difference was not statistically significant (Table I). ATase levels from individuals with a transversion mutation were not different from that found in those without a mutation.

Stratification of the data based upon the median ATase activity in normal tissue (104 fmol/mg protein) indicated that there were significantly more GC→AT transitions in individuals whose ATase activity was below the median than above the median (Table II): 42% (13/31) versus 7% (2/30), respectively (χ² = 8.4, P < 0.005). In a comparable analysis of transversions, there was no difference in the frequency of mutations in individuals whose ATase activity was below or above the median (Table II): 13% (4/31) versus 10% (3/30), respectively (Fishers exact test, P = 1.0). Individuals with low ATase activity in normal tissue (i.e. below the median) were at increased risk of having a GC→AT transition (OR 10.1; 95% CI 1.9–99.0), but not a transversion mutation (OR 1.3; 95% CI 0.2–9.9) in their colorectal tissue (Table II).
ATase protection of colorectal tissue

Table II. Distribution of mutations in the K-ras oncogene by ATase activity in normal and tumour tissue

<table>
<thead>
<tr>
<th>Mutation</th>
<th>ATase activity</th>
<th>No. of subjects with/without mutations</th>
<th>OR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Below median</td>
<td>Above median</td>
</tr>
<tr>
<td>G→A transition Normal + tumour</td>
<td>Normal&lt;sup&gt;a&lt;/sup&gt;</td>
<td>13/18</td>
<td>10.1 (1.9–99.0)</td>
</tr>
<tr>
<td></td>
<td>Normal&lt;sup&gt;b&lt;/sup&gt;</td>
<td>9/19</td>
<td>2.7 (0.6–13.8)</td>
</tr>
<tr>
<td>G→A transition tumour</td>
<td>Normal&lt;sup&gt;c&lt;/sup&gt;</td>
<td>8/19</td>
<td>11.4 (1.2–522.6)</td>
</tr>
<tr>
<td></td>
<td>Tumour&lt;sup&gt;d&lt;/sup&gt;</td>
<td>6/19</td>
<td>3.6 (0.6–39.8)</td>
</tr>
<tr>
<td>G→C/T transversion Normal + tumour</td>
<td>Normal&lt;sup&gt;e&lt;/sup&gt;</td>
<td>4/27</td>
<td>1.3 (0.2–9.9)</td>
</tr>
<tr>
<td></td>
<td>Tumour&lt;sup&gt;f&lt;/sup&gt;</td>
<td>3/25</td>
<td>0.7 (0.1–4.6)</td>
</tr>
</tbody>
</table>

Median ATase activity was 4104, 4126, 4112 and 4127 fmol/mg protein.

(or GC→CG/TA transversions) in individuals whose ATase activity was above or below the median (Table II): 24% (9/28) and 15% (4/27) of individuals with ATase activity above or below the median respectively had a GC→AT transition (χ² = 0.78, P = 0.38). There was no detectable association between the levels of tumour ATase activity and the risk of having a transition or transversion mutation (Table II).

Discussion

Susceptibility to alkylating agent induced carcinogenesis in model systems depends upon the interaction between the extent of exposure (which potentially determines initial levels of DNA damage), DNA repair enzyme capacity (which determines the persistence of such damage) and the extent of cellular proliferation (as a route to the fixation of mutagenic lesions: 10,11). In humans, risk factors that determine whether an individual is susceptible to the carcinogenic effects of alkylating agents are still largely unknown, but given the known function of ATase, the large inter-individual variations in its activity observed in different human tissues may play a role in determining cancer risk (12–14). One study has suggested that lung cancer risk is higher in individuals with low ATase activity as fibroblast ATase activity was lower in lung cancer patients than in healthy controls (15).

In this study, we have found an inverse association between the levels of ATase activity in normal colorectal tissue (i.e. the target tissue) and the risk of having a K-ras mutation in the same tissue. Though this association might have resulted from the disease process itself or the presence of potential confounding factors such as p53 mutations (16), a similar association was also found when we examined the relationship between ATase levels in normal tissue and mutations detected in tumour tissue. Additionally, as we did not detect a relationship between tumour ATase levels and mutational risk, this suggests that the level of ATase activity in normal tissue is indeed a factor involved in determining susceptibility to the development of a K-ras mutation. Further analytical studies, however, are needed to confirm this hypothesis and to show whether individuals with low ATase activity are at increased risk of developing colorectal cancer.

Given that ATase levels may play a role in determining susceptibility to alkylating agent induced carcinogenesis, it becomes increasingly important to be able to characterize those factors, whether genetic or environmental that influence ATase expression. Whether genetic differences may result in inter-individual variations in ATase activity is poorly understood as no functional genotypes have as yet been identified (17). Environmental influences are clearly important, since exposure to a number of agents can either transiently induce (e.g. cigarette smoke, ionising radiation, alkylating agents and 2-acetylaminofluorene: 18–21) or reduce (e.g. alkylating agents and pseudosubstrates: 22–24) ATase expression in different cells and tissues. In a previous study we found an inverse correlation between ATase and O₆-MeG levels in human bladder samples obtained from individuals living in the Nile Delta region of Egypt who are exposed chronically to N-nitroso compounds (9) indicating that this exposure, in part, influences ATase levels. However, in the present sample set, we found no association between current exposure to methylating agents (indicated by the presence or absence of O₆-MeG) and K-ras mutations (8), and also no relationship between O₆-MeG levels and ATase activity (data not shown). This suggests that the observed differences in ATase activity do not simply reflect differences in levels of exposure to alkylating agents.

Our data are consistent with the known action of ATase in repairing lesions (primarily O₆-MeG) that induce GC→AT transitions and suggests that alkylating agent exposure is involved in the aetiology of those tumours which contain a GC→AT transition mutation in the K-ras oncogene. Furthermore, as O₆-MeG is a highly cytotoxic lesion probably as a result of the action of mismatch repair enzymes on O₆-MeG-T mismatches produced upon DNA replication (25), exposure to alkylating agents may also be an aetiologic factor in the mutation of mismatch repair proteins and, consequently, the development of those tumours with microsatellite instability (26). The sources of such alkylating agent exposure have yet to be determined, but it is known that bile acids can be nitrated to give carcinogens (27) and bacterially mediated-endogenous nitration reactions within the colon generate large amounts of compounds that are detected using procedures that detect N-nitroso compounds (28). The colon and rectum are thus potentially exposed chronically to a number of different alkylating agents. A reduction in exposure to these alkylating agents or increased ATase expression may therefore be expected to reduce the incidence of, at least, K-ras mutated colorectal tumours.

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References

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