Reduced MGMT activity in human colorectal adenomas is associated with K-ras GC—AT transition mutations in a population exposed to methylating agents

Nicholas P. Lees1,2, Kathryn L. Harrison2,3, C. Nick Hall1, Geoffrey P. Margison2 and Andrew C. Povey3,4

1Department of Gastrointestinal Surgery, Wythenshawe Hospital, Southmoor Road, Wythenshawe, Manchester M23 9LT, 2Cancer Research UK Carcinogenesis Group, Paterson Institute for Cancer Research, Christie Hospital, Manchester M20 4BX and 3School of Epidemiology and Health Sciences, Medical School, The University of Manchester, Oxford Road, Manchester M13 9PT, UK

To whom correspondence should be addressed
Email: a.povey@man.ac.uk

There is increasing evidence to suggest that O6-alkylguanine DNA-alkyltransferase (MGMT) activity provides protection against alkylating agent induced formation of GC—AT transition mutations in the K-ras oncogene of colorectal tumours. As this mutagenic event occurs during the growth of adenomas, both biomarkers of exposure (N7-methylguanine levels in DNA) and susceptibility (MGMT activity) were measured in biopsy samples obtained from normal and adenomatous tissue from 34 patients with large adenomas (>10 mm in size). There was no correlation between MGMT activity in the adenoma and in matched normal tissue. However, MGMT activity was significantly lower in adenoma tissue than in adjacent normal mucosa (5.18 versus 7.05 fmol/μg DNA, P = 0.01), particularly in men and those whose age was greater than the median. Upon stratification by K-ras mutational status, MGMT activity was lower in adenomas bearing a K-ras GC—AT transition mutation (mean 4.21 fmol/μg DNA) than in adjacent normal tissue (mean 7.7 fmol/μg DNA; P < 0.004). In contrast, there was no significant difference in MGMT activity in adenomas lacking a K-ras GC—AT transition mutation and adjacent normal mucosa. N7-methylguanine levels however did not vary with age, gender, K-ras mutational status or MGMT activity. These results are consistent with the acquisition of K-ras GC—AT transition mutations in adenomas with low MGMT activity as a result of unavoidable exposure to methylating agents.

Introduction

Human exposure to methylating N-nitroso compounds may be an important environmental determinant of individual risk of colorectal neoplasia. A range of different DNA adducts are induced by methylating agents including N7-methylguanine, quantitatively the adduct formed in highest amounts, and the pro-carcinogenic O6-methylguanine. O6-Methylguanine is detectable in DNA from the colorectal mucosa, clearly indicating that human colorectal mucosa is exposed to methylating agents (1,2) with levels tending to be higher in the cancer-prone regions of the large bowel (3). O6-Methylguanine levels will however depend not only on exposure but also upon the level of O6-alkylguanine DNA-alkyltransferase (MGMT), which repairs O6-methylguanine and becomes inactivated in the process (4,5). N7-methylguanine levels in colorectal DNA may reflect better exposure as these adducts are poorly repaired but their levels in colorectal DNA are unknown. The newly developed immunoslot blot (ISB) technique (6) provides a potential method for measuring N7-methylguanine in DNA obtained from small biopsy specimens.

Colorectal tumours can be induced experimentally by treatment with alkylating agents such as 1,2-dimethylhydrazine (7). These tumours frequently contain GC—AT transition mutations in K-ras, which is consistent with the known mutagenic properties of O6-methylguanine (8,9). Furthermore, such GC—AT transition mutations account for the majority of the K-ras mutations seen within human colorectal cancers (CRCs; 10). MGMT provides protection against the toxic, mutagenic and carcinogenic properties of alkylating agents (4,5). In animals treated with 1,2-dimethylhydrazine, over-expression of MGMT reduces the formation of both K-ras GC—AT gene mutations and colorectal tumours (11).

Inter-individual variations in colon MGMT activity are large, being between 2- and 18-fold and 2- and 33-fold in normal and tumour tissue, respectively, as reported in different studies (reviewed in ref. 12). The complete absence of functional MGMT activity in human colorectal tissue has been reported (12). The mechanisms underlying these variations remain incompletely understood.

Most CRCs develop from colorectal mucosa via a benign adenomatous stage (13). Acquisition of a K-ras mutation is a common event during adenoma progression. Whilst a K-ras mutation is seen in only 10% of adenomas <10 mm in diameter, ~50% of adenomas ≥10 mm possess such a mutation, as do a similar proportion of CRCs (14). Low MGMT activity in normal colorectal mucosa has been associated with the occurrence of GC—AT transition mutations in the K-ras oncogene of colorectal cancers (15). Furthermore, methylation of CpG dinucleotides in the CpG island of the promoter region of the MGMT gene, a common event in primary human neoplasia (16) has been associated with both reduced MGMT expression (17,18) and an increased frequency of GC—AT transition mutations in K-ras in colorectal cancers (19,20) and in p53 in colorectal tumours and astrocytomas (21,22).

Hence, this study was designed to examine whether there is an association between MGMT activity and N7-methylguanine levels and the presence or absence of K-ras GC—AT transition mutations in the adenoma itself.

Materials and methods

Human tissues

Human tissue samples were obtained, with the approval of the Local Research Ethics Committee and in accordance with British Regulations, from consenting

Abbreviations: ISB, immunoslot blot; MGMT, O6-alkylguanine DNA-alkyltransferase.
patients undergoing a flexible endoscopic examination of the colon and rectum on clinical grounds in the Department of Gastrointestinal Surgery, Wythenshawe Hospital, Manchester, UK. During the procedure, the patient received sedation with the short-acting benzodiazepine, midazolam (Antigen Pharmaceuticals, Tipperary, Ireland), and supplemental oxygen, as judged necessary by the endoscopist. Air was used for insufflation.

Polyps were treated endoscopically by electrocautery snare or fulguration (‘hot biopsy’) as determined by the endoscopist on clinical grounds. The maximum diameter of polyps was determined immediately after their removal. Patients with pedunculated adenomatous polyps ≥10 mm in diameter formed the subjects of this study. A biopsy was taken from the adenoma (away from the junction with the stalk). Mucosal pinch biopsies were taken ~2 cm away from the base of the polyp, using FB 24 U-1 colonoscopy biopsy forceps (Olympus, Southend-On-Sea, Essex, UK). For each patient one of the normal mucosa biopsies was fixed in Carson’s solution (60% v/v ethanol, 30% v/v chloroform and 10% v/v acetone); all were examined histologically and were found to be normal. The remaining normal mucosa biopsies and the adenoma biopsies were snap frozen and stored at −80°C until an MGMT assay was performed. All polyps were subsequently examined histologically by a consultant pathologist for confirmation of adenomatous nature.

MGMT activity

Cell free extracts prepared from between 5 and 15 mg of normal mucosa (two pooled biopsies per patient) or adenoma tissue were analysed for MGMT activity using calf thymus DNA methylated in vitro with N-nitroso-N-[3H]-methylurea (~20 Ci/mmol) as the substrate (23). MGMT activity was expressed as fmol/μg DNA to avoid the possible effect of variable protein content on apparent MGMT activity expressed per unit protein (24). No significant differences in the study results were noted when MGMT activity was expressed per unit protein.

Results are the mean of quadruplicate determinations for each sample. All matched case-control pairs were assayed simultaneously. Cell free extracts prepared from the human B-lymphoid cell line Raji were assayed for MGMT activity with each batch of mucosal samples as a positive control. The mean coefficient of variation of the quadruplicate MGMT assays for each sample was 7%. The same batch coefficient of variation in MGMT determination based on assays of Raji extracts was 2%, and the different batch coefficient of variation was 11%.

K-ras mutation analysis

Adenoma DNA was extracted from paraffin tissue blocks and screened for K-ras codon 12 and 13 mutations via a restriction site mutation assay (2).

The sequence of all detected mutations was determined using an Amplification Refractory Mutation System (Eliigenic, Zeneca Diagnostics, Alderley Edge, Cheshire, UK) following the manufacturer’s recommendations.

Determination of N7-MeG in colorectal mucosal DNA

Genomic DNA for N7-MeG measurement was extracted from colorectal mucosal biopsies using the Qiagen genomic DNA extraction kit (Qiagen, Crawley, UK) except that the proteinase K and ribonuclease A digestion was carried out overnight at 4°C, followed by 1 h at 37°C. Levels of N7-MeG in the DNA of colorectal mucosa were measured using an ISB technique (4). In brief, sonicated DNA from samples and MNU-methylated CT-DNA standards were alkali treated to open the imidazole ring of N7-MedG adducts in the DNA (70 mM NaOH, at 37°C for 30 min), neutralized and then heat-denatured (5 min, 100°C) and cooled on ice for 10 min to generate single stranded DNA. Samples are then immobilized on nitrocellulose (NC) filters and the slots rinsed. The filter is removed from the support and baked at 80°C for 90 min and was then blocked for 1 h with a milk solution. Primary rabbit polyclonal antibody diluted 1:10 000 in PBS-Tween (0.1% v/v) containing 0.5% fat-free milk powder was applied overnight at 4°C. Following washing, the NC filters were then incubated with goat anti-rabbit IgG horseradish peroxidase conjugate diluted 1:10 000 in PBS-Tween (0.1% v/v) containing 0.5% fat-free milk powder for 1 h at room temperature. The enzymatic activity associated with slots was visualized by bathing the NC filters in chemiluminescent reagent and exposing to X-ray film. The band intensities were quantified by scanning the X-ray films with an optical scanner (Storm 860; Amersham Biosciences, Piscataway, USA). The mean coefficient of variation of the triplicate ISB assays was 9.8% for both cases and controls. The limit of detection is 0.1 μmol N7-MedG/mol dG.

Statistical analysis

The level of significance was tested between tissues by the Wilcoxon signed ranks test for analyses of unmatched samples. A test for analyses of matched samples and the Mann-Whitney two-tailed U test for analyses of unmatched samples. A P value of <0.05 was taken as statistically significant.

Results

MGMT activity in adenomatous tissue and adjacent normal mucosa

MGMT activity was detected in adenoma tissue in 30 of the 34 adenomas and levels ranged between 1.57 and 12.40 fmol/μg

![Fig. 1. MGMT activity in adenomatous tissue and in normal mucosa from the same patient. No significant correlation was detected (r = 0.05, P = 0.79).](https://academic.oup.com/carcin/article-abstract/25/7/1243/2390719)
DNA. MGMT activity was not detected in four adenoma samples. MGMT activity in normal tissue from the same patients ranged from 2.39 to 14.30 fmol/mg DNA. There was no significant correlation between MGMT activity in adenoma tissue and the normal mucosa from the same patient (Figure 1).

MGMT activity was significantly lower in adenoma tumour tissue than in adjacent normal mucosa (Table I), particularly in men and in those patients whose age was greater than the median.

Of the 34 adenomas in which MGMT activity had been determined, DNA suitable for K-ras mutation analysis was available in 26. K-ras codon 12 or 13 mutations were found in 16 adenomas of which 12 were GC→AT transition...

**Table I.** MGMT activity (fmol MGMT/μg DNA) in adenoma tissue and adjacent normal tissue

<table>
<thead>
<tr>
<th>Variable</th>
<th>Strata</th>
<th>n</th>
<th>MGMT activity (mean ± SD)</th>
<th>Difference in MGMT activity (normal-adenoma) (mean; 95% CI)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Adenoma tissue</td>
<td>Adjacent normal tissue</td>
<td></td>
</tr>
<tr>
<td>All</td>
<td></td>
<td>34</td>
<td>5.18 ± 3.26</td>
<td>7.05 ± 2.55</td>
<td>1.88 (0.47–3.29)</td>
</tr>
<tr>
<td>Gender</td>
<td>Male</td>
<td>21</td>
<td>4.71 ± 3.27</td>
<td>7.01 ± 2.20</td>
<td>2.30 (0.39–4.21)</td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>13</td>
<td>5.95 ± 3.23</td>
<td>7.12 ± 3.13</td>
<td>1.12 (–1.13–3.52)</td>
</tr>
<tr>
<td>Age</td>
<td>&lt;71 years (median)</td>
<td>18</td>
<td>5.17 ± 3.40</td>
<td>6.84 ± 2.70</td>
<td>1.67 (–0.48–3.82)</td>
</tr>
<tr>
<td></td>
<td>&gt;71 years</td>
<td>16</td>
<td>5.19 ± 3.20</td>
<td>7.30 ± 2.43</td>
<td>2.11 (0.07–4.16)</td>
</tr>
<tr>
<td>K-ras mutational status of adenoma</td>
<td>GC-AT mutation positive</td>
<td>12</td>
<td>4.21 ± 2.35</td>
<td>7.70 ± 2.83</td>
<td>3.50 (1.04–5.95)</td>
</tr>
<tr>
<td></td>
<td>GC-AT mutation negative</td>
<td>14</td>
<td>5.55 ± 3.21</td>
<td>6.68 ± 2.92</td>
<td>1.13 (–0.85–3.12)</td>
</tr>
</tbody>
</table>

**Fig. 2.** Determination of N7-methylguanine in colorectal DNA. (A) Scan of ISB of human samples. Each well contains 1 μg DNA. Standards, containing varying levels of N7-MeG from 0 to 5.4 fmol/μg DNA, are in columns 1–8 in duplicate. Human DNA samples are in columns 9–22 in triplicates. (B) Standard curve of N7-methylguanine.
Table II. N7-Methylguanine levels in normal tissue from adenoma patients

<table>
<thead>
<tr>
<th>Variable</th>
<th>Strata</th>
<th>n</th>
<th>( \mu \text{mol N7-MeG/mol dG} ) Mean ± SD</th>
<th>Range</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>All subjects</td>
<td></td>
<td>29</td>
<td>0.76 ± 0.95</td>
<td>0–3.71</td>
<td>0.97</td>
</tr>
<tr>
<td>Gender</td>
<td>Male</td>
<td>18</td>
<td>0.77 ± 0.91</td>
<td>0–3.59</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Female</td>
<td>11</td>
<td>0.75 ± 1.06</td>
<td>0–3.71</td>
<td>0.99</td>
</tr>
<tr>
<td>Age</td>
<td>&lt;76 (median)</td>
<td>16</td>
<td>0.76 ± 1.17</td>
<td>0–3.71</td>
<td></td>
</tr>
<tr>
<td></td>
<td>&gt;76</td>
<td>13</td>
<td>0.77 ± 0.62</td>
<td>0–1.80</td>
<td></td>
</tr>
<tr>
<td>K-ras mutational status of adenoma</td>
<td>GC-AT mutation positive</td>
<td>11</td>
<td>1.07 ± 1.02</td>
<td>0.11–3.59</td>
<td>0.36</td>
</tr>
<tr>
<td></td>
<td>GC-AT mutation negative</td>
<td>11</td>
<td>0.64 ± 1.12</td>
<td>0–3.71</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 3. MGMT activity in normal colon mucosa versus N7-methylguanine levels in DNA from the normal colon mucosa. No significant correlation was detected \((r = -0.013, P = 0.92)\).

Discussion

Previously, we reported that although MGMT activity was lower in tumours with GC→AT transition mutations in the K-ras oncogene than in those without this mutation this difference was not statistically significant \((P = 0.36; \text{Table II})\).

N7-methylguanine levels in normal colorectal DNA

N7-methylguanine levels were measured in 29 of the 34 normal tissue samples and detectable levels were found in all but three DNA samples. A typical example of an ISB with a standard curve is shown in Figure 2. N7-methylguanine levels were highly variable, ranging from below the limit of detection to 3.71 \( \mu \text{mol N7-MeG/mol dG} \) indicating at least a 70-fold variation in adduct levels. There was no significant association between N7-methylguanine levels and age or sex of the patient (Table II) and MGMT activity (Figure 3). N7-methylguanine levels in normal DNA were 50% higher in patients with a GC-AT mutation than those without, but this increase was not significant \((P = 0.36; \text{Table II})\).
and MGMT activity need to be better defined.

reduce this pathway, factors affecting both DNA alkylation methylating agents may not be the key factor in the formation exposure may not reflect exposure relevant to the time period study thus provides further evidence that alkylating agents when the mutation occurred or that the level of exposure to risks but we were unable to detect significantly raised adduct levels in patients with a GC

methylating agents. These differing levels of exposure to from normal tissue. N7-methylguanine levels varied at least 70-fold suggesting widely differing levels of exposure to measured levels of N7-methylguanine in colorectal DNA (19–22) as MGMT promoter methylation has been reported to reduce MGMT expression (17,18). Reduced intra-adenoma MGMT activity may thus be a critical factor in the development of GC→AT transition mutations in the K-ras gene. Such results are consistent with the hypothesis that MGMT activity protects adenoma cells against the formation of GC→AT transition mutations in the K-ras gene.

To address the question of which alkylating agent or agents may be implicated in this K-ras mutational activation, we measured levels of N7-methylguanine in colorectal DNA from normal tissue. N7-methylguanine levels varied at least 70-fold suggesting widely differing levels of exposure to methylating agents. These differing levels of exposure to methylating agents will no doubt result in differing mutational risks but we were unable to detect significantly raised adduct levels in patients with a GC→AT transition mutation. This may simply reflect the small sample size, or that current exposure may not reflect exposure relevant to the time period when the mutation occurred or that the level of exposure to methylating agents may not be the key factor in the formation of a K-ras GC→AT transition mutation. Presumably, given sufficient exposure, deficient MGMT activity (and other repair systems) may have an important aetiologic role. The present study thus provides further evidence that alkylating agents play a role in human colorectal carcinogenesis. In order to reduce this pathway, factors affecting both DNA alkylation and MGMT activity need to be better defined.

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References


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