MGMT and MLH1 promoter methylation versus APC, KRAS and BRAF gene mutations in colorectal cancer: indications for distinct pathways and sequence of events

S. de Vogel1,2, M. P. Weijenberg1, J. G. Herman3, K. A. D. Wouters2, A. F. P. M. de Goeij2, P. A. van den Brandt1, A. P. de Briel2 & M. van Engeland2*

Departments of 1Epidemiology; 2Pathology, GROW—School for Oncology and Developmental Biology, Maastricht University, Maastricht, The Netherlands and 3The Sidney Kimmel Comprehensive Cancer Center, Johns Hopkins School of Medicine, Baltimore, USA

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Background: To study how caretaker gene silencing relates to gatekeeper mutations in colorectal cancer (CRC), we investigated whether O6-methylguanine DNA methyltransferase (MGMT) and Human Mut-L Homologue 1 (MLH1) promoter hypermethylation are associated with APC, KRAS and BRAF mutations among 734 CRC patients.

Methods: We compared MGMT hypermethylation with G:C > A:T mutations in APC and KRAS and with the occurrence of such mutations in CpG or non-CpG dinucleotides in APC. We also compared MLH1 hypermethylation with truncating APC mutations and activating KRAS and BRAF mutations.

Results: Only 10% of the tumors showed both MGMT and MLH1 hypermethylation. MGMT hypermethylation occurred more frequently in tumors with G:C > A:T KRAS mutations (55%) compared with those without these mutations (38%), P < 0.001. No such difference was observed for G:C > A:T mutations in APC, regardless of whether mutations occurred in CpG or non-CpG dinucleotides. MLH1 hypermethylation was less common in tumors with APC mutations (P = 0.006) or KRAS mutations (P = 0.001), but was positively associated with BRAF mutations (P < 0.001).

Conclusions: MGMT hypermethylation is associated with G:C > A:T mutations in KRAS, but not in APC, suggesting that MGMT hypermethylation may succeed APC mutations but precedes KRAS mutations in colorectal carcinogenesis. MLH1-hypermethylated tumors harbor fewer APC and KRAS mutations and more BRAF mutations, suggesting that they develop distinctly from an MGMT methylator pathway.

Key words: APC, BRAF, KRAS mutations, CRC, MGMT methylation, MLH1 methylation

Introduction

In addition to mutations in oncogenes and tumor suppressor genes, epigenetic alterations, including aberrant methylation of DNA repair genes, play an important role in the initiation and development of cancer [1]. Although aberrations in these repair genes (also referred to as caretaker genes) do not directly affect cell growth, they can result in a higher rate of mutations in oncogenes and tumor suppressor genes (called gatekeeper genes) [2, 3]. Promoter hypermethylation inhibits the expression of DNA repair genes, occurs in early stages of cancer development and possibly even precedes somatic mutations in the initiation of colorectal cancer (CRC) [4–6]. Hypermethylation of O6-methylguanine DNA methyltransferase (MGMT), for example, has been observed in normal-appearing colorectal tissue adjacent to tumor tissue, which suggests that MGMT methylation may occur before mutations in other key genes early in the multistep process of colorectal carcinogenesis [7, 8]. The O6-MGMT enzyme prevents G:C > A:T point mutations by removing alkyl adducts from the O6 position of guanine [9], and it was suggested that MGMT hypermethylation may lead to G:C > A:T mutations in KRAS [10, 11] and that loss of MGMT may lead to such mutations in PIK3CA [12]. Although an association of G:C > A:T APC mutations with APC hypermethylation and concurrent hypermethylation of MGMT was previously suggested [13], the relation between MGMT hypermethylation exclusively and G:C > A:T mutations in the APC gene has not previously been studied.

Promoter hypermethylation of the mismatch repair gene Human Mut-L Homologue 1 (MLH1) is associated with microsatellite instability and BRAF mutations in CRC [14–18] and also likely to be an early event in colorectal carcinogenesis [5, 19]. It was previously observed that microsatellite-instable
tumors may harbor less aberrations in *KRAS*, *TP53* or *APC*, indicating that these tumors develop through a distinct pathway [20–23]. In this respect, an interesting recent observation was that *MGMT* and *MLH1* promoter hypermethylation were mutually exclusive [24]. These results also indicate the existence of several distinct pathways in CRC, although the sample size used in that study was relatively small.

Because epigenetic silencing of caretaker genes may result in gatekeeper gene mutations, we aimed to investigate whether an association exists between *MGMT* hypermethylation and *G:C > A:T* *APC* mutations and to study how this relates to the association between *MGMT* hypermethylation and *G:C > A:T* *KRAS* mutations. We also assessed whether *MLH1* promoter methylation is associated with mutations in *APC*, *KRAS* and *BRAF* in a large cohort of incident sporadic CRC patients.

**methods**

**study population and tissue samples**

Tissue material was obtained from incident CRC patients from The Netherlands Cohort Study on diet and cancer, which has been described in detail elsewhere [25]. Briefly, this prospective cohort study was initiated in September 1986 and includes 58,279 men and 62,573 women aged 55–69 years and free of disease at baseline, who originated from 204 Dutch municipalities with computerized population registers. The entire cohort is monitored for cancer occurrence by annual record linkage to The Netherlands Cancer Registry (NCR, comprising nine cancer registries in The Netherlands) and to Pathologisch Anatomisch Landelijk Geautomatiseerd Archief (PALGA), a nationwide network and registry of histopathology and cytopathology reports [26, 27].

The PALGA database was used to identify and locate tumor tissue in Dutch pathology laboratories. CRC was classified according to disease site as follows: colon, i.e. proximal and distal colon, rectosigmoid and rectum. At baseline, all cohort members filled out a self-administered food-frequency questionnaire through which information about age, sex and family history of CRC was also obtained. Tumor material of the CRC patients was collected after approval by the ethical review boards of Maastricht University, the NCR and PALGA. In addition, all 54 pathology laboratories in The Netherlands agreed to make relevant tissue samples available from PALGA upon request. These tissue samples were retrieved from August 1999 to December 2001, as described previously [28].

In total, 734 incident CRC patients were identified from a follow-up period of 7.3 years after baseline, of whom a PALGA report of the lesion and possibly by alkyl adduct formation. Because epigenetic silencing of caretaker genes may result in gatekeeper gene mutations, we aimed to investigate whether an association exists between *MGMT* hypermethylation and *G:C > A:T* *APC* mutations and to study how this relates to the association between *MGMT* hypermethylation and *G:C > A:T* *KRAS* mutations. We also assessed whether *MLH1* promoter methylation is associated with mutations in *APC*, *KRAS* and *BRAF* in a large cohort of incident sporadic CRC patients.

**promoter methylation analyses**

DNA methylation in the CpG islands of the *MGMT* and *MLH1* gene promoters was determined by chemical modification of genomic DNA with sodium bisulfite and subsequent methylation-specific PCR (MSP) as described in detail elsewhere [29]. In brief, 500 ng of DNA was denatured by NaOH and modified by sodium bisulfite. DNA samples were then purified using Wizard DNA purification resin (Promega, Leiden, The Netherlands), again treated with NaOH, precipitated with ethanol and resuspended in H2O.

To facilitate MSP analysis on DNA retrieved from formalin-fixed, paraffin-embedded tissue, DNA was first amplified with flanking PCR primers that amplify bisulfite-modified DNA but do not preferentially amplify methylated or unmethylated DNA. The resulting fragment was used as a template for the MSP reaction [30, 31].

All PCRs were carried out with controls for unmethylated alleles (DNA from normal lymphocytes), methylated alleles (normal lymphocyte DNA treated *in vitro* with Sss methyltransferase [New England Biolabs, Ipswich, MA]) and a control without DNA. Ten microliters of each MSP reaction was directly loaded on to nondenaturing 6% polyacrylamide gels, stained with ethidium bromide and visualized under UV illumination. MSP analyses were successfully carried out for 693 patients for the *MGMT* promoter and for 686 patients for the *MLH1* promoter.

**gene mutation analyses**

Mutation analyses of the mutation cluster region (MCR) in *APC* (codons 1286–1520) were carried out as described previously [32]. Briefly, nested PCR was used to amplify the MCR in four overlapping DNA fragments and the purified fragments were sequenced. Two observers independently evaluated the sequence patterns and data entry. One or more fragments of the MCR could not be analyzed completely from 72 CRC patients, leaving 662 patients with complete information on *APC* mutation status for data analysis. Mutations that lead to the introduction of a stop codon (truncating mutations) subsequently lead to functional loss of the *APC* protein. Such functional *APC* mutations were observed in 37% of CRCs [32] and are considered in the current study, as well as frameshift mutations in *APC*. Exon 1 (codons 12 and 13) of the *KRAS* oncogene was analyzed successfully for all 734 patients using nested PCR, followed by direct sequencing of purified fragments, as previously described [28].

*G:C > A:T* mutations (being *G > A* and/or *C > T* mutations) in either of the two genes were also considered, irrespective of the consequence of the mutation. It is important to note that in *APC*, these mutations may occur via two different chemical transformations depending on the orientation of the mutated cytosine or guanine. Methylated cytosines in the context of a CpG dinucleotide are prone to spontaneous deamination [33], whereas non-CpG cytosines are more likely to mutate by alkyl adduct formation (Figure 1).

We also included mutations of the *BRAF* gene in our study, and for this purpose, the common V600E *BRAF* mutation was analyzed as previously described in detail [34].

**statistical analyses**

Data analyses were conducted separately for CRCs with or without promoter hypermethylation of *MGMT* and *MLH1*. First, we evaluated

![Figure 1. G:C > A:T transitions caused by deamination and possibly alklylation in CpG dinucleotides. *Methyl cytosine with neighboring guanine that mutates to thymine primarily by spontaneous deamination and possibly by alkyl adduct formation. †C > T mutations with corresponding G > A mutations on the other DNA strand.](image-url)
the association between MGMT and MLH1 hypermethylation. Then, we compared the prevalence of MGMT hypermethylation with G>C > A:T point mutations in KRAS or in APC (irrespective of leading to a truncation or not) and with the occurrence of BRAF mutations. We also determined whether the percentage of MGMT hypermethylation differed between G>C > A:T mutations in APC that occur in either CpG or non-CpG dinucleotides.

The prevalence of MLH1 hypermethylation was compared with mutations that lead to functional alterations of APC, KRAS and BRAF, i.e. truncating and frameshift APC mutations, activating KRAS mutations and V600E BRAF mutations. In addition, we assessed the relation between BRAF mutations and truncating APC mutations and activating KRAS mutations. Potential differences in prevalence or differences in patient characteristics were tested by chi-square tests or two-sided t-tests where appropriate. Bonferroni correction was applied to correct for multiple testing by adjusting the significance level of $\alpha = 0.05$ according to the number of tests carried out. A total of nine chi-square tests were carried out to compare frequencies of the molecular aberrations. Therefore, a $P$ value threshold of 0.05/9 = 0.006 was used for all these comparisons.

All statistical analyses were carried out with the STATA statistical software package (intercooled STATA, version 9.1).

### Results

Overall, hypermethylation of MGMT and MLH1 was observed in 41% and 22% of the patients, respectively (Table 1). We observed that hypermethylation of both MGMT and MLH1 occurred in only 66 (10%) of the patients with available MSP analyses. Women more often showed MLH1 hypermethylation (26%) compared with men (19%, $P = 0.03$), whereas no sex difference existed for MGMT hypermethylation (Table 1). In addition, patients less frequently had a positive family history of CRC when they showed hypermethylation in MGMT or MLH1, although these differences were only borderline statistically significant. However, this difference was significant when considering patients with hypermethylation in either of the genes since a family history of CRC was observed in 8% of the patients with MGMT and/or MLH1 methylation compared with 13% among patients showing no methylation ($P = 0.03$, data not shown). Whereas MGMT hypermethylation was independent of tumor sublocalization, we observed that MLH1 hypermethylation occurred more often in proximal colon tumors (35%) compared with distal colon (16%), rectosigmoid

### Table 1. Comparison of colorectal cancer patients with or without MGMT and MLH1 promoter hypermethylation

<table>
<thead>
<tr>
<th></th>
<th>Promoter hypermethylation&lt;sup&gt;a&lt;/sup&gt;</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Methylated</td>
<td>Unmethylated</td>
<td>Methylated</td>
<td>Unmethylated</td>
<td></td>
</tr>
<tr>
<td>Number of patients (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age at diagnosis, mean (SD), year</td>
<td>68.0 (4.3)</td>
<td>67.9 (4.3)</td>
<td>68.0 (4.4)</td>
<td>68.0 (4.3)</td>
<td></td>
</tr>
<tr>
<td>$P$ valueb</td>
<td>0.64</td>
<td>0.76</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sex, n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Men</td>
<td>156 (41)</td>
<td>229 (59)</td>
<td>73 (19)</td>
<td>309 (81)</td>
<td></td>
</tr>
<tr>
<td>$P$ value</td>
<td>0.59</td>
<td>0.03</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Women</td>
<td>131 (43)</td>
<td>177 (57)</td>
<td>79 (26)</td>
<td>225 (74)</td>
<td></td>
</tr>
<tr>
<td>Family history of colorectal cancer (% yes)&lt;sup&gt;c&lt;/sup&gt;</td>
<td>22 (8)</td>
<td>47 (12)</td>
<td>10 (7)</td>
<td>62 (12)</td>
<td></td>
</tr>
<tr>
<td>$P$ value</td>
<td>0.10</td>
<td>0.08</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Tumor sublocalization&lt;sup&gt;d&lt;/sup&gt;, n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Proximal colon</td>
<td>95 (41)</td>
<td>137 (59)</td>
<td>80 (35)</td>
<td>150 (65)</td>
<td></td>
</tr>
<tr>
<td>Distal colon</td>
<td>82 (38)</td>
<td>132 (62)</td>
<td>35 (16)</td>
<td>178 (84)</td>
<td></td>
</tr>
<tr>
<td>Rectosigmoid</td>
<td>37 (46)</td>
<td>43 (54)</td>
<td>12 (16)</td>
<td>64 (84)</td>
<td></td>
</tr>
<tr>
<td>Rectum</td>
<td>67 (42)</td>
<td>92 (58)</td>
<td>21 (13)</td>
<td>138 (87)</td>
<td></td>
</tr>
<tr>
<td>$P$ value</td>
<td>0.65</td>
<td>&lt;0.001</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dukes’ stage&lt;sup&gt;e&lt;/sup&gt;, n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>84 (48)</td>
<td>91 (52)</td>
<td>36 (21)</td>
<td>139 (79)</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>87 (39)</td>
<td>138 (61)</td>
<td>52 (23)</td>
<td>172 (77)</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>72 (41)</td>
<td>105 (59)</td>
<td>44 (25)</td>
<td>133 (75)</td>
<td></td>
</tr>
<tr>
<td>D</td>
<td>32 (40)</td>
<td>48 (60)</td>
<td>14 (18)</td>
<td>62 (82)</td>
<td></td>
</tr>
<tr>
<td>$P$ value</td>
<td>0.28</td>
<td>0.63</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Differentiation&lt;sup&gt;f&lt;/sup&gt;, n (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Good</td>
<td>27 (40)</td>
<td>40 (60)</td>
<td>12 (18)</td>
<td>55 (82)</td>
<td></td>
</tr>
<tr>
<td>Moderate</td>
<td>174 (40)</td>
<td>257 (60)</td>
<td>86 (20)</td>
<td>340 (80)</td>
<td></td>
</tr>
<tr>
<td>Poor</td>
<td>41 (41)</td>
<td>58 (59)</td>
<td>31 (32)</td>
<td>67 (68)</td>
<td></td>
</tr>
<tr>
<td>Undifferentiated</td>
<td>2 (29)</td>
<td>5 (71)</td>
<td>2 (29)</td>
<td>5 (71)</td>
<td></td>
</tr>
<tr>
<td>$P$ value</td>
<td>0.93</td>
<td>0.07</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup>Complete data on MGMT and MLH1 hypermethylation were available for 693 and 686 of 734 patients, respectively. Sixty-six patients had hypermethylation of both MGMT and MLH1.

<sup>b</sup>$P$ value for the difference between the methylated and unmethylated groups.

<sup>c</sup>Data on family history of colorectal cancer, tumor sublocalization, Dukes’ stage and differentiation were unavailable for 3, 9, 38 and 93 patients, respectively. MGMT, $O^6$-methylguanine DNA methyltransferase; MLH1, Human Mut-L Homologue 1.
(16%) and rectal tumors (13%, P < 0.001). All other characteristics presented in Table 1 did not substantially differ between cases with or without MGMT and MLH1 hypermethylation.

The relation between MGMT hypermethylation and the occurrence of G:C > A:T mutations in KRAS and APC is shown in Table 2. After Bonferroni correction, a P value threshold of P = 0.006 was used as the significance level. Tumors with G:C > A:T KRAS mutations showed a higher percentage of MGMT hypermethylation (55%) compared with those without G:C > A:T point mutations (38%, P < 0.001). The percentages of MGMT hypermethylation in tumors with or without G:C > A:T mutations in APC did not differ significantly. Since G:C > A:T mutations in APC may also occur via spontaneous deamination when occurring in CpG dinucleotides, we stratified MGMT hypermethylation according to the orientation of these mutations. However, we then observed that the frequency of MGMT hypermethylation was similar in tumors with G:C > A:T APC mutations in either CpG or non-CpG dinucleotides.

We also assessed the relation between MLH1 hypermethylation and the occurrence of truncating or frameshift APC mutations or activating KRAS or BRAF mutations. We observed less MLH1 hypermethylation in patients with a truncating APC mutation (17%) compared with patients without such a mutation (26%, P = 0.006; Table 3). A similar but statistically borderline significant contrast could be observed for APC frameshift mutations since MLH1 hypermethylation occurred in 15% of the tumors with an APC frameshift mutation versus 25% of tumors without frameshift mutation in APC (P = 0.01). Less MLH1 hypermethylation was also observed in patients with activating KRAS mutations compared with cases without these mutations (15% and 26%, respectively, P = 0.001). Conversely, a strong positive association was present between MLH1 hypermethylation and activating BRAF mutations since 47% of the tumors with BRAF mutations showed MLH1 hypermethylation compared with 17% of MLH1 hypermethylation in tumors harboring wild-type BRAF (P < 0.001).

Finally, we observed that BRAF mutations were more common in tumors without APC mutations or without KRAS mutations (P < 0.001 for both associations, respectively; Table 4). No association was observed between BRAF mutations and MGMT promoter methylation (data not shown).

**discussion**

Here, we studied the prevalence of promoter hypermethylation of the DNA repair genes MGMT and MLH1 in relation to somatic mutations in APC, KRAS and BRAF in colorectal carcinogenesis, in a large group (n = 734) of unselected, untreated patients with colorectal cancer. We aimed to assess whether the different DNA repair genes were involved in the pathogenesis of colorectal cancer and whether their promoter methylation was related to specific somatic mutations. The main findings of our study were that MGMT and MLH1 were frequently hypermethylated in colorectal cancer and that the occurrence of G:C > A:T mutations in KRAS and APC was associated with promoter hypermethylation of MGMT and MLH1. Furthermore, MLH1 hypermethylation was more frequent in tumors with activating BRAF mutations.

**Table 2. MGMT promoter hypermethylation versus G:C > A:T mutations in KRAS and APC**

<table>
<thead>
<tr>
<th>MGMT promoter hypermethylation</th>
<th>Kras&lt;sup&gt;8&lt;/sup&gt;</th>
<th>APC&lt;sup&gt;9&lt;/sup&gt;</th>
<th>P value&lt;sup&gt;10&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methylated, n (%)</td>
<td>64 (45)</td>
<td>198 (59)</td>
<td>0.001</td>
</tr>
<tr>
<td>Unmethylated, n (%)</td>
<td>79 (55)</td>
<td>135 (41)</td>
<td>0.006</td>
</tr>
</tbody>
</table>

<sup>8</sup>Complete data on MGMT promoter hypermethylation were available for 693 of 734 patients.

<sup>9</sup>KRAS mutation analysis succeeded for all 734 patients.

<sup>10</sup>APC mutation analyses were available for 662 of 734 patients.

**Table 3. Promoter hypermethylation of MLH1 versus mutations in APC, KRAS or BRAF**

<table>
<thead>
<tr>
<th>MLH1 promoter hypermethylation</th>
<th>APC (truncation)&lt;sup&gt;11&lt;/sup&gt;</th>
<th>APC (frameshift mutation)</th>
<th>KRAS (activation)&lt;sup&gt;12&lt;/sup&gt;</th>
<th>BRAF (activation)&lt;sup&gt;13&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methylated, n (%)</td>
<td>195 (83)</td>
<td>110 (85)</td>
<td>218 (85)</td>
<td>58 (53)</td>
</tr>
<tr>
<td>Unmethylated, n (%)</td>
<td>295 (74)</td>
<td>375 (75)</td>
<td>316 (74)</td>
<td>462 (83)</td>
</tr>
</tbody>
</table>

<sup>11</sup>Complete data on MLH1 promoter hypermethylation was available for 686 of 734 patients.

<sup>12</sup>APC mutation analyses were available for 662 of 734 patients.

<sup>13</sup>KRAS mutation analysis succeeded for all 734 patients.
incident CRC patients. MGMT hypermethylation was associated with G:C > A:T KRAS mutations. However, there was no association between MGMT hypermethylation and G:C > A:T mutations in APC, irrespective of the occurrence of these mutations in CpG or non-CpG dinucleotides. This indicates that G:C > A:T APC mutations are independent of, and possibly occur before, MGMT hypermethylation. Whereas there was a positive association between MLH1 hypermethylation and BRAF mutations, it was inversely associated with APC mutations and KRAS mutations.

These results support the hypothesis that MGMT hypermethylation may lead to somatic mutations in KRAS and that functional loss of MGMT may occur early in colorectal carcinogenesis [8]. This is the first study in which the association between MGMT methylation and G:C > A:T APC mutations was investigated. A possible explanation for the lack of association between these two events may be the orientation of cytosines or guanines, which may ‘mask’ a potential association between MGMT methylation and G:C > A:T mutations. If these mutations occur in CpG dinucleotides, they may also result from spontaneous deamination [33], whereas alkyl adduct formation is more likely the primary cause in non-CpG dinucleotides. MGMT removes alkyl adducts from the O6 position of guanine, and it was previously observed that G:C > A:T mutations are repaired less effectively via MGMT when they occur in CpG dinucleotides [35]. Moreover, it was also observed that MGMT hypermethylation occurred less frequently in tumors with G:C > A:T P53 mutations in CpG dinucleotides compared with such mutations in non-CpG dinucleotides [10]. In order to investigate whether this difference could explain the lack of association with G:C > A:T APC mutations in our study, we stratified the analyses for those mutations occurring in CpG or non-CpG dinucleotides. However, stratification did not reveal a difference in MGMT methylation between these two types of G:C > A:T APC mutations, and this therefore suggests that the association does not exist. MGMT hypermethylation in combination with hypermethylation of APC or MLH1 may be associated with G:C > A:T APC mutations [13]. However, in that study, the association was not studied for MGMT hypermethylation exclusively, and the conclusion was based on rather small number of cases. Moreover, Halford et al. [36] previously investigated the association between O6-MGMT protein expression and G:C > A:T APC mutations. Although the investigators demonstrated a weak association with G:C > A:T mutations in the combination of APC, beta-catenin, KRAS and P53, no association was observed with G:C > A:T mutations in APC exclusively.

In view of these results, a plausible explanation is to assume that in colorectal carcinogenesis, truncating APC mutations may occur before epigenetic silencing of MGMT by promoter hypermethylation. Mutations in KRAS are viewed as occurring in a later stage of colorectal carcinogenesis than APC mutations, and we observed an association between MGMT hypermethylation and KRAS mutations. Esteller et al. [11] previously observed a similar association between MGMT hypermethylation and G:C > A:T mutations in KRAS. Moreover, an association of MGMT hypermethylation with these mutations in the P53 gene, which usually occur in a late stage of colorectal carcinogenesis [10], was observed. We propose that the sequence of events in the multistep model of colorectal carcinogenesis, as proposed by Fearon and Vogelstein [37], may include MGMT methylation as depicted in Figure 2. It should be noted, however, that the molecular end points studied in tumor tissue are cross-sectional data and can therefore only provide indirect evidence for a potential

![Figure 2](https://academic.oup.com/annonc/article-abstract/20/7/1216/344769/1220)

**Figure 2.** MGMT and MLH1 promoter methylation and mutations in APC, KRAS and BRAF in the carcinogenesis of sporadic colorectal cancer. Adapted from Fearon and Vogelstein [37]. Adenomas were not analyzed in the study and were therefore omitted from this model. †P53 mutation status was not analyzed in this study.
sequence of events. Nevertheless, such data provide more insight in colorectal carcinogenesis and may be an important source for new hypotheses about the possible timing of molecular events.

A second observation in the current study was that MLH1 hypermethylation was related to lower frequencies of somatic mutations in APC and KRAS, which suggests that such tumors develop through a distinct genetic pathway. This is in line with the observed high correlation between MLH1 methylation and microsatellite instability in the colorectum [15–17] and its inverse relationship with mutations in other key genes involved in colorectal carcinogenesis [20–23]. In addition, we have previously observed a strong positive association between MLH1 methylation and MSI and that APC and KRAS mutations are rare in MLH1-deficient tumors among CRC patients included in the current study [18, 38]. Moreover, strong associations between MLH1 hypermethylation, MSI, and BRAF mutations were previously observed [14]. In this respect, we demonstrated that BRAF-mutated tumors were strongly associated with MLH1 hypermethylation and moreover that these tumors also harbored fewer mutations in APC and KRAS. These observations confirm previous results showing that BRAF mutations were inversely associated with KRAS mutations and with APC hypermethylation [39, 40]. On the other hand, MGMT promoter hypermethylation was not associated with BRAF mutations and our results as well as observations of a smaller study [24] show that concurrent promoter hypermethylation of MLH1 and MGMT is rare. We therefore suggest that these are additional indications showing that tumors with epigenetic silenced MGMT may arise through a distinct hypermethylation-associated pathway.

Patients with MGMT and/or MLH1 hypermethylation less often had a positive family history of CRC but there were no age differences between patients with or without hypermethylation. This suggests that in these tumors, exogenous factors may have played a larger role in colorectal carcinogenesis compared with tumors that do not develop through a hypermethylated pathway.

We conclude that MGMT inactivation by hypermethylation may cause somatic mutations in KRAS, but not in APC. This suggests that in the process of colorectal carcinogenesis, MGMT hypermethylation may succeed the introduction of APC mutations, but precedes KRAS mutations. Tumors with MLH1 inactivation by hypermethylation seem to develop through a distinct pathway associated with fewer APC and KRAS mutations, and this pathway is possibly distinct from an MGMT methylator pathway. Colorectal carcinogenesis may be more susceptible to the influence of exogenous factors when hypermethylation plays an important role.

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