Hypermethylation of \( O^6 \)-Methylguanine-DNA Methyltransferase Promoter May Predict Nonrecurrence after Chemotherapy in Colorectal Cancer Cases

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ABSTRACT

**Purpose:** Because \( O^6 \)-methylguanine-DNA methyltransferase (MGMT) plays an essential role in repairing DNA damage caused by environmental alkylating chemicals, we were interested in determining whether we could see any obvious changes in the properties of colorectal cancers (CRCs) in which the MGMT gene had been silenced by hypermethylation and hence in which very few MGMT protein molecules were being produced.

**Experimental Design:** We used a methylation-specific PCR assay to determine the methylation status of the MGMT promoter in the DNA molecules extracted from CRC and nontumor tissue samples from 116 patients who had undergone CRC surgery and for whom clinical outcome information was available on file.

**Results:** We found evidence of MGMT promoter hypermethylation in 26 of 90 CRC cases, and we noted that the later the stage at which a tumor was diagnosed, the less likely its MGMT promoter was to be methylated \((P = 0.03, \text{ adjusting for chemotherapy})\), especially for stage D patients \((P = 0.01)\). We also found that CRC patients with unmethylated MGMT promoters were much more likely to experience recurrence within 36 months than patients with hypermethylated MGMT promoters \((\text{crude odds ratio, } 14.0; 95\% \text{ confidence interval, } 2.42–81.01)\). After adjustment for stage, CRC patients with unmethylated MGMT promoters who had been exposed to chemotherapy were found to have a 5.3-fold greater risk of recurrence than those who had no exposure to chemotherapy \((95\% \text{ confidence interval, } 1.15–30.92)\).

**Conclusions:** Hypermethylation of the MGMT promoter may be predictive of a low risk of recurrence in CRC patients receiving adjuvant chemotherapy.

INTRODUCTION

CRC is a significant cause of morbidity and mortality in industrialized societies. Despite major advances in the diagnosis and treatment of CRC, mortality has changed very little over the last three decades, with an overall 5-year survival of around 40%. Considerable effort has therefore been devoted to obtain a better understanding of the molecular and biological mechanisms involved in the development and progression of CRCs. The identification of such molecular markers may help to target patients for whom specific adjunct therapies may be appropriate, thereby increasing survival times.

MGMT, also known as \( O^6 \)-alkylguanine-DNA alkyltransferase, is a DNA repair protein that removes mutagenic and cytotoxic adducts from \( O^6 \)-guanine in DNA (1). This protein specifically removes alkyl groups from the \( O^6 \)-position of guanine and transfers them to an active center of its own; the net effect is that the protein is inactivated, and the guanine in DNA is restored to its original state (2). The MGMT protein is therefore widely regarded as a major contributor to the protection of cells against the mutagenic, carcinogenic, and cytotoxic effects of DNA-alkylating agents. Inactivation of the MGMT gene is likely to result in the retention of methylated adducts at the \( O^6 \)-position of guanine. When this occurs, thymidine is likely to be incorporated opposite \( O^6 \)-MeG during DNA copying, and if the resulting mismatched thymidine is not removed and replaced by cytosine by the MMR system, the outcome is likely to be a GC to AT transition mutation at the site in question. An alternative outcome involves cells whose MGMT genes have been inactivated being channeled into the apoptotic pathway (3–6).

MGMT expression levels vary widely between normal and tumor tissues samples (7, 8), as well as between different cells.
in a single tumor (9), and there are indications that some 20% of human tumor cell lines produce virtually no MGMT protein at all (10). This is a particularly interesting observation, given that loss of MGMT expression is almost never accompanied by evidence that the gene has undergone deletion, rearrangement, or any other form of mutational change (11). Thus there is good reason to suppose that the MGMT gene is susceptible to epigenetic regulation, and this is borne out by recent evidence showing that hypermethylation of its promoter leads to the MGMT gene being silenced in numerous human cancer cases [including colorectal cancer, stomach cancer, and certain other forms of cancer (12–15)]. A recent study showed that MGMT promoter hypermethylation was associated with significantly increased survival in patients with diffuse large B-cell lymphoma after treatment with multidrug regimens including cyclophosphamide (16).

In this paper, we examine the relationships between reductions in the intracellular levels of the MGMT protein that result from hypermethylation of the MGMT promoter in CRC and disease outcome, and we use multivariate analysis to investigate possible roles of MGMT in cancer recurrence and in disease-free and overall survival after curative surgery.

MATERIALS AND METHODS

Patients and Tumor Specimens. Paired primary tumor and normal tissue specimens from 116 CRC patients were obtained from the Department of Surgery, Okayama University Medical Hospital and Okayama Saiseikai Hospital. All patients underwent curative surgery without prior chemo- or radiotherapy between 1994 and 1999. Tumors had to be large enough to provide adequate amounts of tissues for genetic analysis without compromising pathological diagnosis. Tissues from nonneoplastic areas of the tumor and from normal mucosa were placed on ice immediately upon removal from the patient and then frozen at −80°C until DNA could be extracted from them. For survival analysis, only tumors from patients who had undergone curative resection were considered (n = 90). Advanced-stage patients classified as Dukes’ stage C and D received chemotherapy with oral fluoropyrimidines for 1 year postoperatively. All patients attended the outpatient clinic every 3 months for the first 2 years postoperatively and every 6 months thereafter for follow-up, and the data so obtained were used in our prognostic analysis. Potential confounders of the relationship between MGMT methylation and prognosis (age, sex, administration of chemotherapy, and Dukes’ stage) were measured and controlled in the analysis when necessary.

Bisulfite Treatment and MSP. The methylation status of the MGMT promoter region was evaluated by performing MSP analysis of genomic DNA. MSP analysis distinguishes unmethylated from methylated alleles in the MGMT region on the basis of the nucleotide sequence changes that are produced after bisulfite treatment of DNA. This process converts unmethylated, but not methylated, cytosine to uracil; and it is possible to carry out PCR using primers specifically designed to determine DNA methylation status. Bisulfite treatment was performed using a CpGenome DNA Modification Kit (Intergen Co., New York, NY). Primer sequences for "methylated" and "unmethylated" MSP for the MGMT promoter region were: (a) MGMT-MS (5'-TTTTCAGCCTTCGTAGGTTTTCGC-3') and MGMT-MAS (GCACCTTACCAGAACGAGC-3'), and (b) MGMT-US (5'-TTTTGTGTTTGTGTTTGTGTTTGTGT-3') and MGMT-UAS (5'-AACCTCACCAGCACAACAGAC-3'), generating fragment lengths of 81 and 93 bp, respectively (13). PCR was performed with 0.2 μM of each primer, 0.1 mM deoxynucleotide triphosphates, 1.25 units of platinum Taq DNA polymerase (Invitrogen, Carlsbad, CA), and approximately 50 ng of sodium bisulfite-modified DNA. Conditions for PCR amplification were as follows: 94°C for 2 min; 35 cycles of 94°C for 30 s, 59°C for 30 s, and 72°C for 30 s; and finally, 4 min at 72°C. PCR products were run in 2.5% agarose gels. As a positive control for methylated alleles, we used DNA treated in vitro with SSO methyltransferase (New England Biolabs, Inc., Beverly, MA). DNA from human sperm was used as a negative control for methylated genes. All analyses were conducted in duplicate.

MSI Analysis. Two mononucleotide markers, 14 dinucleotide markers, and 1 tetranucleotide marker were used to test for MSI. BAT25, BAT26, D2S123, D5S107, D5S346, D7S480, D7S522, D8S117, D8S254, D8S258, D17S2561, D17S960, D17S250, D18S35, D18S58, and MYCL were used for MSI analysis as described previously (17). Briefly, PCR was performed in 50-μl reaction mixtures containing 0.3 μM each oligonucleotide primer pair (one end-labeled with Texas Red), 200 μM each deoxynucleotide triphosphate, 5 μM of 10× PCR buffer, 1.25 units of Taq polymerase (AmpliTaqGold; PerkinElmer, Foster City, CA), and approximately 100 ng of DNA. After denaturation in formaldehyde at 95°C for 5 min, the amplified PCR products were electrophoresed on a 6% LongRanger-6.1 urea gel on a Hitachi Autosequencer SQ 5500 and analyzed by FRAGLYS version 2 software (Hitachi, Inc., Tokyo, Japan). After MSI analysis, CRCs were classified into three groups: (a) group 1, MSS with no MSI at any of the loci examined; (b) group 2, MSI-L with <40% of the loci examined exhibiting MSI; and (c) group 3, MSI-H with >40% of the loci examined exhibiting MSI (17).

Immunohistochemical Analysis. Immunohistochemical staining for the MGMT protein was carried out on tissue samples from 45 cases using the avidin biotin-peroxidase complex method. After deparaffinization and hydration, slides were immersed in EDTA buffer (pH 8.0), irradiated in a microwave oven for 15 min, and sonicated for 2 min. Slides were then incubated with primary anti-MGMT antiserum (clone mt3.1; PharMingen, Fremont, CA) for 3 h (12). Diaminobenzidine was used as a chromogen, and nuclei were counterstained with hematoxylin. Inflammatory cells in the lamina propria served as internal positive controls.

Statistical Analysis. Statistical end points in our uni- and multivariate analysis were disease-free and overall survival from the date of curative surgery, together with CRC recurrence status at 36 months. Survival analysis was conducted with right censoring of patients who died from other diseases or showed no evidence of recurrence. The probability of disease-free and overall survival was plotted over time using the Cox proportional hazards model, and differences between groups were tested with maximum likelihood statistics, adjusting for potential confounders. Disease-free survival was emphasized in this
Hypermethylation of MGMT and Colorectal Cancers

RESULTS

We studied a total of 96 patients with CRC, all of whom were subjected to detailed analysis that included careful methylation analysis of their MGMT promotors (Fig. 1). The MSP data for 6 patients were inconclusive and were therefore excluded, leaving only 90 to be used for prognostic analysis. The characteristics of patients with CRC in relation to the methylation status of their MGMT promotors are shown in Table 1. Methylation of MGMT promotor was detected in 26 of 90 tumors (29%), a value that is roughly the same as those reported elsewhere (13, 18). In the 24 patients whose cancers recurred, the mean time from diagnosis to the onset of recurrent disease was 20.0 months (SD = 10.6 months). There were 59 patients who had either remained disease-free or experienced a recurrence by 36 months. We used disease-free survival at 36 months as a study outcome because most patients were followed for at least this long, and all but one of those recurring had been so diagnosed within this time frame. Classifying the one late-recurring patient as nonrecurrent in our analysis had only a minimal effect on the results of our study. Of the 24 patients with curative treatments who experienced recurrence (i.e., who had a disease-free interval greater than 0), 12 (50%) had done so between 5 and 16 months, whereas the other 12 had so between 19 and 39 months. We describe the first group of patients as early recurrence patients and the second group as late recurrence patients.

Table 1 shows that in univariate analysis, MGMT methylation status was significantly related to chemotherapy, stage, MSI-L, and moderate or poorly differentiated carcinoma. As shown in Table 1, increasing stage at diagnosis was associated with an increased likelihood that MGMT would be unmethylated (P = 0.0002 by two-tailed γ test). Adjusting for the significant association between chemotherapy and MGMT methylation status, Dukes’ C patients were at 2.7-fold greater risk (P = 0.16) of having unmethylated MGMT promotors, whereas all Dukes’ D patients had unmethylated MGMT promotors resulting in an infinite OR (P = 0.01; Table 2).

Tables 3 and 4 show that recurrence was significantly less common in patients whose MGMT promotors were methylated, with only 1 of 15 such patients experiencing recurrent disease. Conversely, after adjusting for stage, recurrence appeared to be much more likely (95% CI, 1.86–infinity) in chemotherapy patients with unmethylated MGMT promotors (Table 3) than in those whose MGMT promotors had been methylated. Among patients with unmethylated MGMT promotors, those undergoing chemotherapy were also significantly more likely to experience a recurrence (adjusted OR, 5.3; 95% CI, 1.15–30.92; Table 4).

Patients receiving or not receiving chemotherapy were combined in the analysis shown in Table 5, which presents both the crude OR of recurrence for MGMT status and the OR after adjustment for chemotherapy status, sex, and stage. After adjusting for these factors, patients with unmethylated MGMT promotors were at 4.4-fold greater risk of recurrence within 36 months of surgery. The 95% CI for the crude OR excluded the null value, whereas that for the adjusted OR did not.

Fig. 2 shows recurrence-free survival separately for patients with Dukes’ A and B (Fig. 2A) and Dukes’ C and D
None of the 10 Dukes C and D patients who had methylated MGMT promoters experienced recurrence, whereas 19 of the 40 with unmethylated MGMT promoters did. Adjusting for chemotherapy status, sex, and stage revealed that patients with unmethylated MGMT promoters were at a 5.6-fold increased risk of recurrence (95% CI, 0.73–42.68; \( P = 0.10 \)) and a 1.3-fold increased risk of death (95% CI, 0.27–6.47). Stage was significantly related to both outcomes. Hazard ratios for stage should be interpreted as the ratio of increase per stage increase (Dukes’ A–D). The hazard ratio for sex represents the increased risk of recurrence and death for males compared with females. Patients receiving chemotherapy were at 2.6-fold increased risk of recurrence (95% CI, 3.0–12.11; \( P = 0.21 \)) and a 1.3-fold increased risk of death (95% CI, 3.9–14.58; \( P = 0.16 \)).

### Table 2  Relationship between MGMT methylation status and tumor stage at diagnosis, adjusting for chemotherapy\(^a\)

<table>
<thead>
<tr>
<th>Methylated status</th>
<th>Dukes’ stage</th>
<th>No. (%)</th>
<th>Crude OR (95% CI)</th>
<th>Adjusted OR (95% CI)(^a)</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hypermethylated</td>
<td>A(^b)</td>
<td>8 (57.1)</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>8 (30.8)</td>
<td>3.0 (0.80–12.11)</td>
<td>2.4 (0.62–10.10)</td>
<td>0.21</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>10 (25.6)</td>
<td>3.9 (1.09–14.58)</td>
<td>2.7 (0.69–11.03)</td>
<td>0.16</td>
</tr>
<tr>
<td></td>
<td>D</td>
<td>0 (0)</td>
<td>( \approx (5.43–\infty) )</td>
<td>( \approx (3.32–\infty) )</td>
<td>0.01(^c)</td>
</tr>
<tr>
<td>Unmethylated</td>
<td>A(^b)</td>
<td>6 (42.9)</td>
<td></td>
<td>1.0</td>
<td></td>
</tr>
<tr>
<td></td>
<td>B</td>
<td>18 (69.2)</td>
<td></td>
<td>2.4 (0.62–10.10)</td>
<td>0.21</td>
</tr>
<tr>
<td></td>
<td>C</td>
<td>29 (74.4)</td>
<td></td>
<td>2.7 (0.69–11.03)</td>
<td>0.16</td>
</tr>
<tr>
<td></td>
<td>D</td>
<td>11 (100)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

\(^a\) Adjusted for chemotherapy status using maximum likelihood estimates.  
\(^b\) Referent.  
\(^c\) Likelihood ratio test used because of zero cell. \( P = 0.03 \) for overall association of stage and methylation status after adjustment for chemotherapy.

### Table 3  ORs of cancer recurrence at 36 months among Dukes’ A–D patients for MGMT methylation status according to chemotherapy status\(^a\)

<table>
<thead>
<tr>
<th>Recurrence at 36 months</th>
<th>Chemotherapy not given</th>
<th>Chemotherapy administered</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MGMT Methylated(^d)</td>
<td>MGMT Unmethylated</td>
</tr>
<tr>
<td>No</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>No. (%)</td>
<td>10 (90.9)</td>
<td>4 (100)</td>
</tr>
<tr>
<td>Yes. (%)</td>
<td>1 (9.1)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

\(^a\) Patient recurring after 36 months was counted as nonrecurrence; patients with noncurative operations were excluded.  
\(^b\) ORs and 95% CIs were adjusted for stage.  
\(^c\) Likelihood ratio \( P \).  
\(^d\) Referent.  
\(^e\) Maximum likelihood ORs and CIs were calculated because of zero cell.  

### Table 4  ORs of cancer recurrence at 36 months among Dukes’ A–D patients for administration of chemotherapy by MGMT methylation status\(^a\)

<table>
<thead>
<tr>
<th>Recurrence at 36 months</th>
<th>MGMT methylated</th>
<th>MGMT unmethylated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Chemotherapy</td>
<td>Chemotherapy</td>
</tr>
<tr>
<td>No(^d)</td>
<td>Yes</td>
<td>No</td>
</tr>
<tr>
<td>No. (%)</td>
<td>10 (90.9)</td>
<td>4 (100.0)</td>
</tr>
<tr>
<td>Yes. (%)</td>
<td>1 (9.1)</td>
<td>0 (0)</td>
</tr>
</tbody>
</table>

\(^a\) Patient recurring after 36 months was counted as nonrecurrence; patients with noncurative operations were excluded.  
\(^b\) ORs and 95% CIs were adjusted for stage.  
\(^c\) Likelihood ratio \( P \).  
\(^d\) Referent.  
\(^e\) Maximum likelihood ORs and CIs were calculated because of zero cell.
greater risk of recurrence and 1.2-fold increased risk of death, taking into account the other relevant factors; however, as with the relationship of recurrence to sex, the relationship of recurrence to chemotherapy was not quite statistically significant.

The presence of aberrant hypermethylation correlated well with loss of MGMT immunoreactivity (P < 0.001, Fisher’s exact test), and there was no case that was negative for methylation in which MGMT did not stain immunohistochemically, with the stain almost invariably being detected in the nucleus.

**DISCUSSION**

This study demonstrates for the first time that hypermethylation of the promoter region of the well-studied DNA repair enzyme MGMT is inversely related to Dukes’ stage in CRC patients. In our univariate analysis, MGMT methylation status was significantly related to the stage and differentiation of CRC, suggesting that MGMT methylation status was strongly related to tumor recurrence and hence had the potential to be a useful prognostic factor for CRC patients. Among the characteristics examined, chemotherapy and Dukes’ stage of disease turned out to be significantly related to recurrence. Because they were also related to MGMT status, we adjusted both the ORs and multivariate survival analysis for these factors. After adjustment for stage, patients with an unmethylated MGMT promoter who underwent chemotherapy turned out to have a significantly (and substantially) greater risk of recurrence. Thus, for example, 22 of the 44 patients who had an unmethylated MGMT promoter experienced recurrence, whereas only 1 of 15 patients with a methylated MGMT promoter experienced recurrence. The crude OR was 14.0 (95% CI, 2.42–81.01), and the adjusted OR was 4.4 (95% CI, 0.48–97.81) when sex, stage, and chemotherapy were taken into account. The lack of statistical significance after adjustment almost certainly reflects the small size of our study and the fact that four factors were included in these statistical models. Adjustment for these factors was important, especially in the case of stage and chemotherapy, which were both related to MGMT status and recurrence. Chemotherapy and stage were also linked, primarily because patients at advanced stages were more likely to receive chemotherapy. Nevertheless, hypermethylation of the MGMT promoter appeared to be associated with a greatly reduced likelihood of tumor recurrence in this patient series.

DNA methylation changes have long been suspected of involvement in human colon cancer. Endogenous nitrosation of amino precursors (e.g., proline) occurs in the human body after ingestion of quantities of nitrate and amines consistent with normal dietary intakes. Nitrosated compounds from dietary nitrates produced in the proximal colon by bacteria or by nitrosation of amines and amines derived from protein catabolism have been detected in the large intestine, where they are believed to act as alkylating agents (19–22). In CRCs, the pro-
mutagenic events that lead to O\textsuperscript{6}-MeG formation can be caused by alkylating agents.

Because MGMT is a DNA repair protein that removes promutagenic O\textsuperscript{6}-lesions from O\textsuperscript{6}-MeG in DNA (1), it seems likely that loss of function of the MGMT gene through either mutation or hypermethylation of its promoter will lead to O\textsuperscript{6}-MeG remaining in DNA and, as a result, promote tumorigenesis via GC to AT transition mutations and generation of K-ras and p53 mutation of the sort that are frequently encountered in CRC (14, 22). Thus, loss of function of the MGMT gene through hypermethylation of its promoter may well lead to an increased risk of CRC in affected individuals.

According to a recent analysis of MSI in CRC in our laboratory, the MSI-L phenotype was found in a high proportion (up to 50%) of early CRC cases but in only about 20% of more advanced cases (17). Because hypermethylation of MGMT and the MSI-L phenotype appear to be significantly correlated in both the present study and an earlier one (12), it may be that loss of MGMT activity leads to a procarcinogenic environment that may be important in the early stages of colorectal tumorigenesis. This observation is consistent with a study by Esteller et al. (22) on the part played by MGMT hypermethylation in the formation of colon adenomas. O\textsuperscript{6}-MeG has also been found in the DNA of normal human colon epithelium adjacent to CRCs (23). Thus the loss of MGMT function may occur at a very early step in CRC carcinogenesis. Cells that lack the MGMT protein may then have a selective growth advantage such that they continue to proliferate, whereas normal cells are continually subjected to delayed cell cycling, depending on the time required for sensing and repairing DNA damage.

With time, however, progressive DNA damage may be sufficient to trigger apoptosis with the help of the MMR system, so that MGMT methylation would no longer confer a growth advantage. With continuing exposure to endogenous or exogenous methylating pressure, it is likely that there will be altered gene expression patterns among cancer cells, permitting the emergence of clones resistant to these agents. Either reactivation of MGMT or MMR inactivation could explain this resistance. Alternatively, there may be selection for subclones without MGMT methylation in heterogeneous tumors.

It is interesting that the reduction in recurrence associated with hypermethylation of the MGMT promoter was most evident among patients who were undergoing chemotherapy. In patients with unmethylated MGMT promoters, chemotherapy led to a significant increase in the rate of recurrence as judged by both the crude and adjusted ORs controlled for stage. It is also interesting to note that the MGMT-methylated group of patients who were receiving chemotherapy showed signs of a significant increase in disease-free survival as judged by the log-rank test (P = 0.02) and Wilcoxon’s rank-sum test (P = 0.03).\textsuperscript{3}

5FU is the most commonly used postoperative adjuvant chemotherapy for CRC patients, and it is sometimes used in conjunction with agents that potentiate sensitivity to 5FU. The relationship between MGMT function and 5FU is unclear, however. For example, Bibby et al. (24) studied the effects of deleting MGMT by examining the effects of the pseudosubstrate O\textsuperscript{6}-BG on the antitumor activity of 5FU and the alkylating agents 2-chloroethoxy-1-nitrosoarene and 1,3-bis(2-chloroethoxy)1-nitrosoarene, and they detected significant enhancement of the growth-retarding effects of O\textsuperscript{6}-BG during treatment of mice carrying human colon cancer xenografts with any one of the three test agents mentioned above. One possibility is that the effect of 5FU chemotherapy is magnified whenever MGMT function is lost (e.g., via hypermethylation), in which case the decreased recurrence rate associated with MGMT methylation observed in the present study is easily explained. Another possibility is, of course, that colorectal tumors with methylated MGMT promoters are less aggressive than tumors with unmethylated promoters.

Our results suggest that CRC patients whose MGMT promoter is unmethylated and who therefore have high levels of MGMT protein activity may not be ideal candidates for 5FU-based adjuvant chemotherapy. One way around this problem may involve administering chemicals such as O\textsuperscript{6}-BG that act to reduce MGMT activity to patients expressing high levels of the MGMT protein if 5FU treatment is decided upon. Research is necessary to determine whether or not a combination regimen of this sort will help to reduce recurrence rates in a group of CRC patients whose MGMT promoters are unmethylated. Our study points out the dangers associated with a simplistic understanding of the role of hypermethylation of specific genes in tumorigenesis. Therefore, we must be careful in the use of agents such as DNA methylase inhibitors to CRC patients whose MGMT promoters are methylated.

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\textsuperscript{3} T. Nagasaka, G. B. Sharp, and N. Matsubara, unpublished observations.


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