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Mutually Exclusive Promoter Hypermethylation Patterns of *hMLH1* and *O*^{6}*-Methylguanine DNA Methyltransferase* in Colorectal Cancer

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Hypermethylation of CpG islands in gene promoter regions is an important mechanism of gene inactivation in cancer. Many cellular pathways, including DNA repair, are inactivated by this type of epigenetic lesion, resulting in proposed mutator phenotypes. Promoter hypermethylation of *hMLH1* has been implicated in a subset of colorectal cancers that show microsatellite instability (MSI). Transcriptional silencing of *O*^{6}*-methylguanine DNA methyltransferase* (*MGMT*) has also been described in a variety of neoplasms and has been associated with a consequent mutational spectrum. We investigated the relationship between *hMLH1* promoter hypermethylation and *MGMT* promoter hypermethylation in 110 colorectal cancers using methylation-specific polymerase chain reaction. Expression of hMLH1 and MGMT was assessed by immunohistochemistry. MSI testing was performed using the National Cancer Institute consensus panel of five microsatellite markers. Promoter hypermethylation of *hMLH1* was detected in 12% of tumors. This was significantly associated with the MSI-high phenotype ($P < 0.01$) and loss of hMLH1 expression ($P < 0.01$). Methylation of the *MGMT* promoter was detected in 43% of tumors, which were mostly microsatellite stable or MSI-low ($P = 0.041$) and showed loss of MGMT expression ($P < 0.01$). We demonstrated an inverse relationship between *hMLH1* promoter hypermethylation and *MGMT* promoter hypermethylation ($P = 0.041$), suggesting that a number of distinct hypermethylation-associated pathways may exist in colorectal cancer. ***(J Mol Diagn 2006, 8:68–75; DOI: 10.2353/jmoldx.2006.050084)***

Tumorigenesis in humans is a multistep process, reflecting an accumulation of genetic changes that lead to the progressive transformation of normal cells into increasingly malignant derivatives.[1] The causes of such genetic alterations are multifactorial, with exogenous and endogenous compounds known to induce a variety of genetic alterations, including deletions, insertions, and base substitutions.[2] Aberrations in DNA repair, giving rise to proposed mutator phenotypes, are hypothesized to contribute to the accumulation of mutations.[3]

The first well-characterized mutator pathway in human cancer involves abnormalities in DNA mismatch repair (MMR) proteins.[4–6] Germline alterations in MMR genes (*hMLH1, hMSH2, hMSH6*, and *hPMS2*) are responsible for the majority of hereditary nonpolyposis colorectal cancer cases.[7–11] MMR-deficient tumors exhibit increased random point mutation rates and deletions or insertions in microsatellite and repetitive sequences, referred to as microsatellite instability (MSI).[12] Because microsatellites and repetitive sequences are frequently present in coding regions, instability can manifest as frameshift mutations that inactivate a variety of genes, including genes that suppress tumor formation, eg, *APC*, *transforming growth factor-βRII*, *hMSH2*, and *caspase-5*.[13,14] This mutator phenotype is also present in 10 to 15% of sporadic cases of colorectal cancer (CRC), in which hMLH1 expression is lost not through mutation but through epigenetic silencing by promoter hypermethylation.[15,16]

Promoter hypermethylation-associated silencing of *O*^{6}*-methylguanine DNA methyltransferase* (MGMT) has been proposed to cause another mutator pathway more prevalent than MSI.[17] MGMT is a DNA repair protein that removes promutagenic and cytotoxic adducts from O^6-guanine in DNA. It protects cells against potentially deleterious effects of endogenously and exogenously produced O^6-alkylating agents, including mutation, sister chromatid exchanges, recombination, and chromosomal aberrations.[18] Loss of MGMT function during tumorigenesis has been suggested to result in mutations in key cancer-related genes. Silencing of *MGMT* has been shown to be strongly linked to the presence of G to A transition mutations in *K-ras*, the most frequent type of mutation, in colon,[19] gastric,[20] and gall bladder cancers.[21] A similar association has been described with

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Table 1. Primer Sequences for MSP[15]

Gene		Sense	Antisense	Size (bp)
hMLH1	U	5′-TTT TGATGTAGATGTTTTATTAGGGTTGT-3′	5′-ACCACCTCATCATAACTACCCACA-3′	124
	M	5′-ACGTAGACGTTTTATTAGGGTCGC-3′	5′-CCTCATCGTAACTACCCGCG-3′	115
MGMT	U	5′-TTTGTGTTTTGATGTTTGTAGGTTTTTGT-3′	5′-AACTCCACACTCTTCCAAAAACAAAACA-3′	93
	M	5′-TTTCGACGTTCGTAGGTTTTCGC-3′	5′-GCACTCTTCCGAAAACGAAACG-3′	81

M, methylated; U, unmethylated.

C:G to T:A transitions in *p53* of colorectal carcinomas,[22] astrocytomas,[23] gliomas,[24] and non-small-cell lung cancers.[25] Although genetic alterations of *MGMT* are rarely detected in human cancers,[26] *MGMT* silencing associated with promoter hypermethylation has been documented in a variety of different tumor types and is observed in approximately 20 to 40% of CRC cases.[27–29]

The functional interaction between MGMT and DNA mismatch repair has been extensively characterized in transgenic animals and cell lines.[30–32] These latter studies collectively proposed a model whereby functional mismatch repair over an unrepaired alkylated residue, occurring, for example, in a MGMT-deficient background, results in double-strand breaks (DSBs) and potentially chromosomal alterations. Although the occurrence of each respective mutator phenotype has been described, no specific interrelation has been demonstrated. In this study, we examined the promoter hypermethylation and immunohistochemical status of hMLH1 and MGMT in an unselected series of sporadic colorectal carcinomas and correlated these findings with clinical and pathological data.

Materials and Methods

Tumor Specimens

We randomly selected 110 colorectal cancers from patients attending the SVUH Centre for Colorectal Disease. Histologically normal mucosa, remote from the tumor, was also obtained. Each cancer case selected was sporadic, ie, lacking family history or clinical evidence of familial adenomatous polyposis or hereditary nonpolyposis colorectal cancer. Ethical approval to conduct this study was granted by the St. Vincent's University Hospital Ethics and Medical Research Committee.

Histopathological Features

Conventional histopathological parameters, including the American Joint Committee on Cancer/International Union Against Cancer tumor-node-metastasis stage, tumor type, and differentiation, were assessed using a standard reporting template. Tumors were typed as adenocarcinomas or mucinous adenocarcinomas according to the criteria of the World Health Organization.[33] Differentiation (well/moderate or poor) was determined following the World Health Organization guidelines.[33] Additional histological parameters assessed included lymphovascular invasion,[34] perineural invasion,[35] tumor margin,[36] and peritoneal involvement.[37,38]

Microsatellite Marker Analysis

MSI analysis was performed on DNA from the snap-frozen samples for the paired tumor and normal adjacent mucosa using fluorescently labeled primers for the National Cancer Institute consensus panel of microsatellite markers (D2S123, D5S346, D17S250, BAT25, and BAT26). Size variations were analyzed by capillary gel electrophoresis (ABI 310; Applied BioSystems, Foster City, CA). Tumors were scored as MSI-high (MSI-H) if two or more of the five markers showed instability, MSI-low (MSI-L) if only one of these markers showed instability, or stable (MSS) if none of the markers showed any size variation.

Methylation-Specific Polymerase Chain Reaction (PCR)

DNA methylation patterns in the CpG islands of *hMLH1* and *MGMT* genes were determined using methylation-specific PCR (MSP).[39] Briefly, <2 μg of genomic DNA from tumor and paired non-neoplastic tissue was denatured by treatment with NaOH and modified by sodium bisulfite. DNA samples were purified using Wizard DNA purification resin (Promega, Madison, WI), treated with NaOH, precipitated with ethanol, and resuspended in deionized water. The primer sequences used are given in Table 1. PCR products were separated on 2.5% agarose gels and visualized under UV illumination.

Immunohistochemistry

hMLH1 Expression

Immunostaining for hMLH1 was performed on 5-μm sections of formalin-fixed paraffin-embedded tissues. Sections were dewaxed in xylene and rehydrated through graded alcohol to water. Endogenous peroxidase activity was quenched by incubating the slides in 3% hydrogen peroxide for 7 minutes. Antigen retrieval was performed by immersing sections in 10 mmol/L citrate buffer (pH 6.0) and heating in a domestic microwave oven (800 W) at full power for 5 minutes, followed by boiling in a pressure cooker for 4 minutes. Sections were left for 20 minutes in buffer to cool to room temperature.

Tissues were stained for hMLH1 using the DAKO Envision kit (DAKO A/S, Glostrup, Denmark) according to the manufacturer's instructions, with the primary antibody against hMLH1 (clone G168-728; PharMingen, San Diego, CA) applied at a dilution of 1:75 for 2 hours. Positive staining was visualized with 3,3-diaminobenzidine sub-

strate solution. Sections were counterstained with Mayer's hematoxylin. Tumor cells were judged to be negative for hMLH1 only if they lacked nuclear staining in a section in which stromal or inflammatory cell nuclei stained positive (Figure 1D).

MGMT Expression

Tissue microarrays were assembled from the formalin-fixed paraffin-embedded tissues using a 0.6-mm-diameter punch (Beecher Instruments, Silver Spring, MD). Four cores were taken from the tumor and the paired normal adjacent mucosa for each case. Sections (5 μm) were cut from each tissue microarray block and used for MGMT immunohistochemistry. Sections were dewaxed and endogenous peroxidase quenched as before. Sections were immersed in boiling citrate buffer in a pressure cooker and then boiled for 5 minutes at full pressure (103 kPa). Sections were stained for MGMT using the avidin-biotin method (Vectastain Elite, Vector Laboratories, Burlingame, CA) with the primary antibody against MGMT (clone mT3.1; Neomarkers, Fremont, CA) applied at a dilution of 1:100 overnight at 4°C. Staining was visualized with 3,3-diaminobenzidine and hematoxylin counterstain as before. Only strong nuclear staining was regarded as indicative of MGMT protein expression (Figure 1A). Loss of MGMT expression was defined as either weak nuclear staining (Figure 1B) or complete absence of nuclear staining (Figure 1C).

Statistical Analyses

Fisher's exact test, χ^2 test, and Mann-Whitney *U*-test were used, as appropriate, to test statistical significance. All calculations were performed using the SPSS 11.0 for Windows statistical software package (SPSS, Inc., Chicago, IL). All reported *P* values are two tailed.

Results

Promoter Hypermethylation and Relationship to MSI and Immunohistochemical Expression

Tables 2 and 3 outline the primary clinicopathological and molecular features of the cohort, respectively. Ten tumors (9%) displayed a MSI-H phenotype and 97 (88%) had no evidence of microsatellite instability. Us-

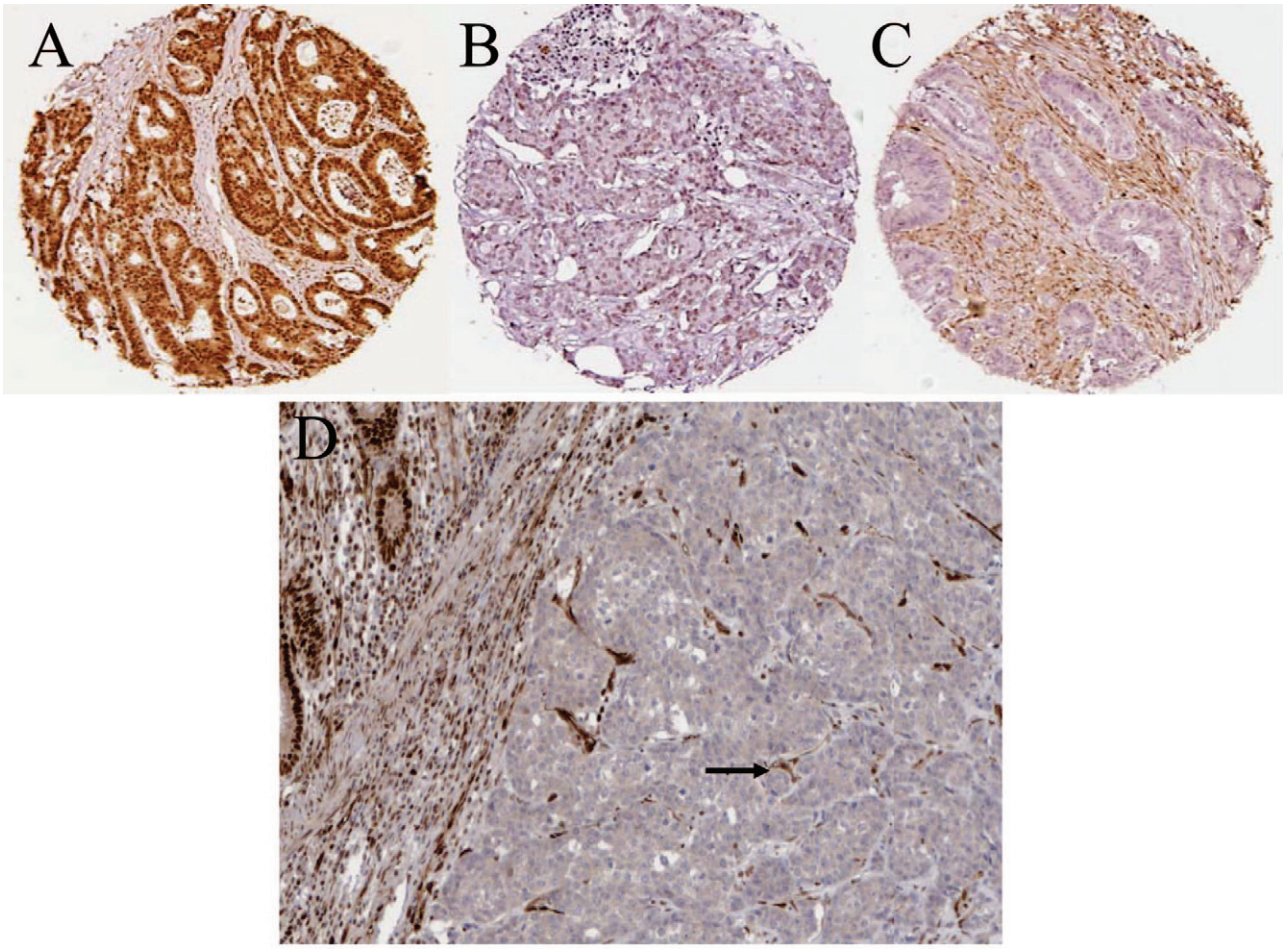

Figure 1. Immunohistochemical staining patterns for MGMT (**A–C**) and hMLH1. **A:** Tumor with positive staining; **B:** tumor showing weak nuclear staining; **C:** tumor showing absence of nuclear staining; **D:** tumor showing loss of hMLH1 expression. Intratumoral endothelial cells (**arrow**) act as an internal positive control. An adjacent area of normal mucosa, staining positive for hMLH1, is present in the **upper left corner**. Magnification, ×100.

ing the Bethesda panel of microsatellite markers, only three MSI-L tumors (3%) were detected. Immunohistochemical absence of an MMR protein was described in nine cases (8%). Absence of hMLH1 (Figure 1D) was described in eight cases (seven of which were MSI-H), absence of hMLH1 and hMSH2 in one case (an 86-year-old male with a poorly differentiated cecal cancer, MSI-H), and absence of hMSH2 in one case (a 70-year-old male with a moderately differentiated cecal cancer, MSI-H). MSI status was therefore 90% sensitive and 99% specific as an indicator of MMR function characterized immunohistochemically.

Promoter hypermethylation of *hMLH1* was detected in 12% (13 of 110) of tumors (Figure 2). This was significantly associated with a MSI-H phenotype ($P < 0.001$). A positive association was observed between *hMLH1* promoter hypermethylation and absence of hMLH1 expression as assayed by immunohistochemistry ($P < 0.001$). Six of 13 cases exhibiting hMLH1 promoter hypermethylation did not show absence of hMLH1 by immunohistochemistry or any level of microsatellite instability. In one-half of these cases (three of six) hMLH1 promoter hypermethylation was also detected in paired normal adjacent mucosa.

Hypermethylation of the *MGMT* promoter was detected in 43% (47 of 110) of tumors and was significantly associated with the MSS/MSI-L phenotype ($P = 0.041$). We found 18% of cases (20 of 110) with complete absence of MGMT expression (Figure 1C), with 18 of 20 displaying *MGMT* promoter hypermethylation. In 15% of cases (17 of 110), weak nuclear staining was seen (Figure 1B); more than one-half of these cases (9 of 17) showed evidence of MGMT promoter hypermethylation. Combining those cases with complete absence and weak nuclear staining gave 37 cases with loss of MGMT expression. Of these 37, 27 (73%) exhibited promoter hypermethylation at this locus. There was no evidence of promoter hypermethylation of MGMT in 57% of cases (63 of 110). MGMT was expressed in 84% (53 of 63) of these cases, with the majority (7 of 10) of the negative cases in this group showing weak nuclear staining and incomplete absence of MGMT staining. Absence of MGMT was thus significantly associated with *MGMT* promoter hypermethylation ($P < 0.001$).

Table 2. Clinicopathological Characteristics of 110 Patients with Colorectal Cancer

Patient characteristics	
Mean age (years [±SD])	69 (±11)
Gender	
Male	52 (47%)
Female	58 (53%)
Tumor site	
Rectum/left colon	74 (66%)
Right colon	36 (33%)
Tumor differentiation	
Well/moderate	84 (74%)
Poor	26 (24%)
Angiolymphatic invasion	
Absent	62 (56%)
Present	48 (44%)
Perineural invasion	
Absent	91 (83%)
Present	19 (17%)
Maximum tumor size (cm [±SD])	4.7 (±1.9)
Tumor stage	
A(T2, N0)	2
B(T3, N0)	32
(T4, N0)	11
C(T2, N1)	2
(T3, N1)	21
(T4, N1)	7
(T2, N2)	7
(T3, N2)	9
(T4, N2)	5
D(T1–4, N1–2, M1)	14

Table 3. Frequencies of Molecular and Immunohistochemical Features in Tumors of 110 Colorectal Cancer Patients

Variable	Number
Microsatellite instability	
MSI-H	10
MSI-L	3
MSS	97
Immunohistochemistry	
Absent hMLH1	9
Absent hMSH2	1
Absent MGMT	37
Promoter hypermethylation	
hMLH1	13
MGMT	47

Relationship between Promoter Hypermethylation Status and Clinicopathological Features

hMLH1 promoter hypermethylation was statistically significantly associated with proximal location ($P = 0.027$), poor differentiation ($P = 0.015$), lower incidence of lymph node metastasis ($P = 0.035$), and large tumor size ($P = 0.025$). Similar trends were described for absence of hMLH1 protein expression: proximal location ($P = 0.056$), lower incidence of lymph node metastasis ($P = 0.078$), and large tumor size ($P = 0.066$). Associations between promoter hypermethylation status and pathological/molecular features are given in Table 4.

Although tumors displaying *MGMT* promoter hypermethylation were more likely to have an expansile margin ($P = 0.105$), no associations with the clinical or pathological variables examined were statistically significant. Absence of MGMT protein expression demonstrated sim-

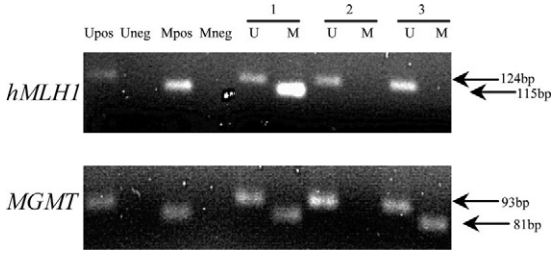

Figure 2. Methylation analysis of *hMLH1* and *MGMT* promoters. MSP was performed with primers specific for methylated (M) and unmethylated (U) regions. Product sizes: hMLH1 unmethylated, 124 bp; hMLH1 methylated, 115 bp; MGMT unmethylated, 93 bp; and MGMT methylated, 81 bp.

Table 4. Association between Promoter Hypermethylation and Pathological, Molecular, and Immunohistochemical Features in 110 Colorectal Cancers

Variable	*hMLH1* hypermethylation			*MGMT* hypermethylation		
	U (n = 97)	M (n = 13)	P value	U (n = 63)	M (n = 47)	P value
Immunohistochemistry						
hMLH1						
Present	95	6	<0.001	56	45	0.30
Absent	2	7		7	2	
MGMT						
Present	61	12	0.057	53	20	<0.001
Absent	36	1		10	27	
MSI						
MSI-H	2	8	<0.001	9	1	0.041
MSI-L/MSS	95	5		54	46	
Hypermethylation						
hMLH1						
Present				11	2	0.041
Absent				52	45	
Tumor site						
Rectum/left colon	69	5	0.027	42	32	1.0
Right colon	28	8		21	15	
Tumor differentiation						
Well/moderate	78	6	0.015	49	35	0.65
Poor	19	7		14	12	
Tumor margin						
Expansile	31	6	0.35	17	20	0.11
Infiltrative	66	7		46	27	
Nodal metastases						
N0	41	10	0.035	28	23	0.70
N1/2	56	3		35	24	
Peritoneal involvement						
No	78	9	0.47	52	35	0.35
Yes	19	4		11	12	
Size (cm [±SD])	4.6 (±1.7)	6.1 (±2.5)	0.025	4.8 (±2.0)	4.6 (1.6)	0.85

M, methylated; U, unmethylated.

ilar nonsignificant clinical and pathological trends as MGMT promoter hypermethylation, with only peritoneal involvement (P = 0.047) reaching significance. Associations between immunohistochemical staining patterns and pathological/molecular features are given in Table 5.

Interrelationship between hMLH1 and MGMT Promoter Hypermethylation Statuses

Of this cohort of 110 cases, two tumors displayed promoter hypermethylation at both loci. *hMLH1* and *MGMT* promoter hypermethylation consequently showed a statistically significant inverse association (P = 0.041). This exclusive pattern was reflected at the immunohistochemical level with only one tumor displaying concomitant loss of both proteins.

Discussion

Although the individual instances of *hMLH1* and *MGMT* promoter hypermethylation, at 12 and 43%, respectively, and their association with gene silencing are well described in colorectal cancer,[27,29,40] we demonstrate a specific relationship between the patterns of promoter hypermethylation of these two genes.

Tumors characterized on this basis are known to be molecularly and clinicopathologically distinct. Similar to previous descriptions,[15,16,19,26,41–44] tumors in this cohort displaying *hMLH1* promoter hypermethylation are more likely to be MSI-H, to be right-sided, to be of poor differentiation, and to have lower levels of nodal metastasis. The phenotypic characteristics of this group are distinct from those tumors displaying *MGMT* promoter hypermethylation, which tend to be clinically and pathologically somewhat more variable, as determined in this study and by others.[29]

The interplay between *MGMT* and MMR functions has been extensively documented in transgenic mice, including MLH1/MGMT−/− double knockout animals, as well as in cell lines.[32] The current view regarding the cellular result of an unrepaired O^6-meG:C is that replication results in an O^6-meG:T mismatch (or possibly an O^6-meG:C ambiguous pair). In the next round of replication, this results in an A:T transition mutation and again to an O^6-meG:C pair or an O^6-meG:T mismatch. The O^6-meG:T mismatch is recognized and initiates MMR that creates a gapped duplex after incision of the newly replicated strand. Because O^6-meG remains in the template, this process may be repeated in a futile repair loop that eventually results in DSBs that are intermediates in both apoptotic and recombinogenic pathways.[31,45] DSBs have been demonstrated to frequently induce various sorts of chromosomal aberrations, including aneuploidy, loss of heterozygosity, and chromosomal translocations—events that are all intimately associated with carcinogenesis[46–49] (Figure 3).

Importantly, in the only such study to date in a mammalian MGMT-deficient cell line, the frequency of recom-

Table 5. Association between Immunohistochemical Staining Patterns of hMLH1 and MGMT and Pathological and Molecular Features in 110 Colorectal Cancers

Variable	hMLH1 staining			MGMT staining		
	Present (n = 101)	Absent (n = 9)	P value	Present (n = 73)	Absent (n = 37)	P value
MSI						
MSI-H	2	8	<0.001	9	1	0.16
MSI-L/MSS	99	1		64	36	
Tumor site						
Rectum/left colon	71	3	0.056	49	25	1.0
Right colon	30	6		24	12	
Tumor differentiation						
Well/moderate	79	5	0.21	57	27	0.64
Poor	22	4		16	10	
Tumor margin						
Expansile	31	6	0.059	22	15	0.29
Infiltrative	70	3		51	22	
Nodal metastases						
N0	44	7	0.078	35	16	0.69
N1/2	57	2		38	21	
Peritoneal involvement						
No	80	7	1.0	62	25	0.047
Yes	21	2		11	12	
Size (cm [±SD])	4.6 (±1.7)	6.3 (±2.9)	1.0	4.8 (±1.9)	4.6 (1.7)	0.90

binogenic to apoptotic events resulting from the presence of O^6-meG residues is relatively high, on the order of 175:1.[50] Thus, the likelihood of cytogenetic change is greater than that of programmed cell death, particularly because the tumor is not specifically being confronted with an alkylating agent. In cells lacking MMR function, DSBs would not be proposed to occur in response to a persistent O^6-meG residue. Because the recombinogenic effects of O^6-meG requires MMR, the potential for genetic instability in an *MGMT*-deficient background is potentiated by functional MMR. Consistent with this, DSBs have been demonstrated to occur in MGMT-deficient/MMR-proficient cells after treatment with an O^6-alkylating agent but not in MMR-deficient cells, regardless of their MGMT functional status.[51] Similarly, the higher relative occurrence of cytogenetic alterations reported in MMR-proficient compared with MMR-deficient colorectal tumors is consistent with this model.[52]

Thus the largely exclusive relationship of *MGMT* and *hMLH1* hypermethylation and expression that we report here may reflect a positive selective pressure for the retention of MMR function in an MGMT-deficient background. This nominally constitutes the preservation of a tumor suppressor function during tumor progression. In the context of deficient MGMT function, functional MMR, through potentiation of genetic instability, promotes tumorigenesis. We hypothesize that no similar selective pressure would exist against loss of MGMT in an MMR-deficient background and that this may in part explain the occasional occurrence of cases lacking both proteins.

Although statistically significant, promoter hypermethylation status was not an absolute indicator of protein expression as assayed by immunohistochemistry for either hMLH1 or MGMT. This may explain the fact that this inverse relationship has not been specifically noted in previous studies in which promoter hypermethylation but not immunohistochemical status of genes, including *hMLH1* and *MGMT*, have been characterized. In particular, some tumors displaying promoter hypermethylation retained protein expression, possibly because of heterogeneous cell populations in the tumor. Because of the

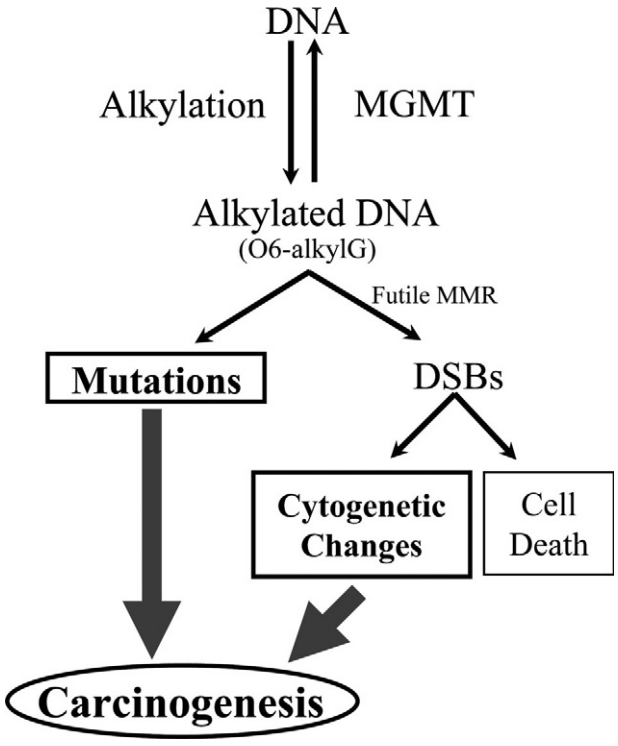

Figure 3. Schematic of the consequences of attempted mismatch repair over an unrepaired alkylated residue in an MGMT-deficient background. Alkylation of DNA in the absence of MGMT function will result in transition mutations, regardless of mismatch repair proficiency. These lesions, in the presence of proficient mismatch repair, will give rise to futile MMR cycles, resulting in the generation of DSBs in DNA. These DSBs may potentially induce various sorts of chromosomal aberrations, including aneuploidy, loss of heterozygosity, and chromosomal translocations.

high sensitivity of PCR-based techniques, hypermethylation could potentially be observed, even if only representative of a subset of tumor cells. Also, the inactivation of either hMLH1 or MGMT protein expression secondary to non-hypermethylation-associated mechanisms, although infrequent, has been described.[26,53–55]

The use of alkylating agent-based therapy in the subpopulation of cancer patients exhibiting *MGMT* promoter hypermethylation has been previously proposed.[56,57] The expected sensitivity of MGMT-deficient cancers to alkylating agent-based therapies has been partially confounded by the prediction from experimental data of the existence of a chemo-resistant subpopulation of MGMT−/MMR− patients. The present data suggest that such a subpopulation may, in fact, be minimal.

That hMLH1 and MGMT inactivation in sporadic CRC is usually associated with promoter hypermethylation consequently results in the existence of promoter hypermethylation-associated mutator phenotypes. The observations reported here propose that these phenotypes are distinct and exclusive and that, in an MGMT-deficient background, tumorigenesis may be driven both by chromosomal alterations dependent on intact MMR function as well as by transition mutations in genes such as *p53* and *K-ras*, which are independent of MMR function.

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