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Advances in Brief

Inactivation of the DNA Repair Gene O^6-*Methylguanine-DNA Methyltransferase* by Promoter Hypermethylation Is Associated with G to A Mutations in *K-ras* in Colorectal Tumorigenesis[1]

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Abstract

O^6*-Methylguanine DNA methyltransferase (MGMT) is a DNA repair protein that removes mutagenic and cytotoxic adducts from the O^6 position of guanine. O^6-Methylguanine mispairs with thymine during replication, and if the adduct is not removed, this results in conversion from a guanine-cytosine pair to an adenine-thymine pair. *In vitro* assays show that MGMT expression avoids G to A mutations and *MGMT* transgenic mice are protected against G to A transitions at *ras* genes. We have recently demonstrated that the *MGMT* gene is silenced by promoter methylation in many human tumors, including colorectal carcinomas. To study the relevance of defective *MGMT* function by aberrant methylation in relation to the presence of *K-ras* mutations, we studied 244 colorectal tumor samples for *MGMT* promoter hypermethylation and K-ras mutational status. Our results show a clear association between the inactivation of *MGMT* by promoter hypermethylation and the appearance of G to A mutations at K-ras: 71% (36 of 51) of the tumors displaying this particular type of mutation had abnormal *MGMT* methylation, whereas only 32% (12 of 37) of those with other K-ras mutations not involving G to A transitions and 35% (55 of 156) of the tumors without *K-ras* mutations demonstrated *MGMT* methylation ($P = 0.002$). In addition, *MGMT* loss associated with hypermethylation was observed in the small adenomas, including those that do not yet contain *K-ras* mutations. Hypermethylation of other genes such as $p16^{INK4a}$ and $p14^{ARF}$ was not associated with either MGMT hypermethylation or *K-ras* mutation. Our data suggest that epigenetic silencing of *MGMT* by promoter hypermethylation may lead to a particular genetic change in human cancer, specifically G to A transitions in the *K-ras* oncogene.

Introduction

Although *ras* mutation is the most common oncogenic alteration in human cancer (1, 2), the incidence of *K-ras* activation varies widely among carcinomas. *K-ras* mutation is rare in human primary breast carcinomas but occurs in approximately one-half of colorectal carcinomas. *ras* oncogenes are transforming after single-point mutations within their coding sequences. Mutations in naturally occurring *ras* oncogenes have been localized in codons 12, 13, 59, and 61. Alteration of codon 12, GGT, is the most common change. Substitution of the wild-type glycine 12 by any other amino acid results in oncogenic activation of this molecule (1, 2).

The mutational spectrum of critical genes in cancer is often not completely random. The tumor suppressor *p53*, the most commonly mutated gene in human cancer, commonly undergoes C to T changes from deamination of methylated cytosines (3). However, in the case of *K-ras*, the absence of a CpG dinucleotide at codon 12 prevents this alteration on either strand. In contrast, *K-ras* mutations often result from G to A transitions. In animal models, chemical carcinogens can target *ras* oncogene sequences. Some modified bases are highly mutagenic because of their miscoding properties, as is the case for O^6-methylguanine, an adduct produced by several carcinogens, including *N*-MNU.[3] O^6-methylguanine is read as adenine by DNA polymerases, thus leading to the frequent generation of G to A transitions in *K-ras* (4). Avoidance of this mutagenic effect is directly related to the presence of a functional DNA repair protein, MGMT (5). MGMT removes alkyl groups, as well as larger adducts involving chloroethylations, at the O^6 position of guanine in a reaction that inactivates one *MGMT* molecule for each lesion repaired. *In vitro* assays also show that endogenous *MGMT* expression protects mammalian cell lines from spontaneous G:C to A:T transitions in the *aprt* gene (6). Further insight into the role of *MGMT* as a keeper of genome integrity comes from animal models. Transgenic mice overexpressing *MGMT* are protected against O^6-methylguanine-DNA adducts caused by MNU (7) and against G to A mutations in *K-ras* in aberrant colorectal crypt foci (8) and lung tumors (9) in mice. No studies connecting these events in human cancer have been reported.

Genetic defects in *MGMT* have not been found in cancer, but hypermethylation of the *MGMT* CpG island as the cause of *MGMT* transcriptional silencing in cell lines defective in O^6-methylguanine repair has been recognized (10–13). Recently, we have reported that the *MGMT* gene is epigenetically inactivated by promoter hypermethylation in many primary tumor types with specific patterns (12). Although this change is not present in breast carcinomas, in which *K-ras* mutations are extremely rare, it occurs in approximately 40% of cases of colorectal carcinomas associated with the loss of MGMT expression (12, 14) and is also frequent in non-small cell lung carcinoma (12). In the present study, we examined whether the epigenetic silencing of *MGMT* might be linked to the presence of *K-ras* mutations in human colorectal tumorigenesis. We determined the *MGMT* methylation status and the presence and type of *K-ras* mutations in codons 12 and 13 in a large collection of primary colorectal carcinomas and adenomas. Our data show that *MGMT* promoter hypermethylation is an early event in human colorectal tumorigenesis, independent of the aberrant methylation of other genes, and linked to the appearance of G to A mutations in the *K-ras* oncogene.

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[3] The abbreviations used are: MNU, methylnitrosourea; MGMT, O^6-methylguanine DNA methyltransferase; RT-PCR, reverse transcription-PCR; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.

Materials and Methods

Tumor Samples. Initially, 79 colorectal carcinomas and 65 colorectal adenomas were obtained from surgical resection specimens of surgical patients at the Johns Hopkins Hospital. Specimen collection procedures were approved by the Joint Committee on Clinical Investigation (Institutional Review Board) of the Johns Hopkins University School of Medicine. Subsequently, 120 additional colorectal carcinoma samples were obtained from surgical patients at the Hospital de Sant Pau in Barcelona between July 1991 and July 1993 under the supervision of Dr. Gabriel Capella. The study protocol was approved by the Ethics Committee. This last set of samples has been previously characterized for *K-ras* and p53 mutations (15) and $p14^{ARF}$ promoter hypermethylation (16). All of the samples were frozen in liquid nitrogen immediately after resection and stored at −70°C until processing. DNA was extracted by standard methods.

Methylation-specific PCR. DNA methylation patterns in the CpG island of the *MGMT* gene were determined by chemical modification of the unmethylated, but not the methylated, cytosines to uracil, and subsequent PCR using primers specific for either the methylated or the modified unmethylated DNA (17). Primer sequences of *MGMT* were for the unmethylated reaction 5′-TTT GTG TTT TGA TGT TTG TAG GTT TTT GT-3′ (upper primer) and 5′-AAC TCC ACA CTC TTC CAA AAA CAA AAC A-3′ (lower primer) and for the methylated reaction 5′-TTT CGA CGT TCG TAG GTT TTC GC-3′ (upper primer) and 5′-GCA CTC TTC CGA AAA CGA AAC G-3′ (lower primer). The annealing temperature was 59°C. Placental DNA treated *in vitro* with Sss I methyltransferase (New England Biolabs) was used as positive control for methylated alleles of *MGMT*, and DNA from normal lymphocytes was used as negative control for methylated alleles of *MGMT*.

Briefly, 1 μg of DNA was denatured by NaOH and modified by sodium bisulfite. DNA samples were then purified using Wizard DNA purification resin (Promega), again treated with NaOH, precipitated with ethanol, and resuspended in water. Controls without DNA were performed for each set of PCR. Ten μl of each PCR reaction was directly loaded onto nondenaturing 6% polyacrylamide gels, stained with ethidium bromide, and visualized under UV illumination.

Reverse Transcription-PCR. RT-PCR was performed as described previously (16), using 3 μg of total cellular RNA to generate cDNA. One hundred ng of this cDNA were amplified by PCR with primers for exon 5′-GCC GGC TCT TCA CCA TCC CG-3′ and exon 5′-GCT GCA GAC CAC TCT GTG GCA CG- 3′ of *MGMT*, which amplify a 211-bp product spanning sequence 339–527 from GenBank accession number M29971. RT-PCR for GAPDH served as a positive control (16). Ten μl of each PCR reaction was directly loaded onto nondenaturing 6% polyacrylamide gels, stained with ethidium bromide, and visualized under UV illumination. The colorectal cancer cell lines HT-29 and RKO, unmethylated at the *MGMT* promoter (12, 13), and *MGMT* proficient were used. The colorectal cell line SW48, hypermethylated at the *MGMT* promoter that lacks *MGMT* expression, (12) was used as negative control.

Detection of *K-ras* Mutations. Mutations at codons 12 and 13 of the *K-ras* gene were detected and characterized by an artificial RFLP/PCR approach (18) and mutant allele-specific amplification (19). Mutations were confirmed by direct cycle sequencing of the PCR products using the AmpliCycle Sequencing kit (Perkin-Elmer, Branchburg, NJ).

Results

MGMT Promoter Hypermethylation and the Occurrence and Type of *K-ras* Mutations. Among 244 colorectal lesions, 179 carcinomas, and 65 adenomas, 103 (42%) demonstrated hypermethylation of *MGMT*. Representative examples of the methylation analysis in colorectal carcinomas and adenomas are shown in Fig. 1*A*. Of these 244 samples, 88 (36%) had mutant *K-ras*. These two events were related because 48 (55%) of the 88 tumors with mutant *K-ras* had methylated *MGMT*, whereas only 55 (35%) of the 156 tumors without *K-ras* mutations had methylated *MGMT* (Fischer's exact test, two-tailed, $P = 0.003$).

One explanation for this association might be that *K-ras* alterations predispose to hypermethylation of genes including *MGMT* because *ras* activity may lead to increases in DNA methyltransferase (20). To address this issue, we have also examined the methylation of other genes hypermethylated in colorectal cancer. *K-ras* mutations were independent of the promoter methylation status of the tumor suppressor genes $p16^{INK4a}$ or $p14^{ARF}$, previously analyzed in a subset of these tumors (16). *MGMT* promoter hypermethylation was also not associated with aberrant methylation of either $p16^{INK4a}$ or $p14^{ARF}$ (16), showing the independence of these events.

Alternatively, an association between *MGMT* promoter hypermethylation and *K-ras* mutations could result from epigenetic inactivation of *MGMT* leading to *K-ras* mutation through the lack of O^6-methylguanine repair. This relationship would be predicted to result in predominantly G to A transitions. Our data clearly support this latter mechanism: 71% (36 of 51) of tumors with G to A mutation in *K-ras* showed *MGMT* epigenetic inactivation, whereas only 32% (12 of 37) of the tumors with other *K-ras* mutations and 35% (55 of 156) of those without *K-ras* mutations exhibited aberrant methylation of the *MGMT* promoter. *K-ras* G to A transitions in codon 12 *versus* codon 13 had a similar distribution of *MGMT* aberrant methylation, 69% (29 of 42) *versus* 78% (7 of 9), respectively. Fig. 1*B* displays in a graphic way the distribution of *MGMT* promoter hypermethylation according to the *K-ras* status. The frequency of *MGMT* methylation in the G to A mutation group was statistically different from tumors with non-G to A *K-ras* mutations (Fischer's exact test two-tailed, $P < 0.0005$) or tumors without *K-ras* mutations (Fischer's exact test two-tailed, $P < 0.0001$). Thus, *MGMT* methylation is tightly linked only to the presence of G to A mutations in *K-ras* and not to other *K-ras* mutations.

Timing of MGMT Hypermethylation-associated Inactivation. To further explore the role for *MGMT* deficiency in the genesis of G to A *K-ras* mutations, we examined the timing of both events. If the inactivation of *MGMT* leads to G to A mutations in *K-ras*, it would be expected that the loss of *MGMT* expression would precede *K-ras* alterations in colorectal tumorigenesis. Although *MGMT* promoter hypermethylation has been associated with a loss of *MGMT* function in primary colorectal carcinomas (12, 14) and cancer cell lines (10–13), we confirmed this tight correlation examining the expression of *MGMT* using RT-PCR in colorectal adenomas. Ten colorectal adenomas without *MGMT* methylation expressed high levels of *MGMT* mRNA, whereas 19 polyps with *MGMT* methylation expressed very little ($n = 5$) or no detectable *MGMT* mRNA ($n = 14$; Fig. 1*C*). Thus, just as in the invasive tumors, transcriptional loss was associated with *MGMT* hypermethylation (12, 14). To further demonstrate that the promoter methylation leads to loss of *MGMT* expression, the treatment of the colorectal cell line SW48—methylated at *MGMT* and without *MGMT* expression—with the demethylating agent 5-aza-2′-deoxycytidine restored *MGMT* expression (Fig. 1*C*).

Within the colorectal lesions described above, we then investigated these changes in relation to stage of the disease. *MGMT* methylation was present in equal frequency in small adenomas <1 cm (9 of 21, 43%), large adenomas ≥1 cm (23 of 44, 52%), and carcinomas (71 of 179, 40%), which suggests that this change occurs early in neoplastic progression. However, in large adenomas and carcinomas, hypermethylation of *MGMT* was associated with G to A mutations in *K-ras* (9 of 12 large adenomas and 27 of 39 carcinomas with G to A mutations had *MGMT* methylation), whereas in small adenomas, no G to A mutations were observed, even in the 9 adenomas with *MGMT* methylation. Thus, *MGMT* promoter hypermethylation (and its consequence, *MGMT* transcriptional inactivation) is an early event in colorectal tumorigenesis, often appearing before the development of large adenomas, and this event precedes the appearance of *K-ras* mutations in colorectal tumors.

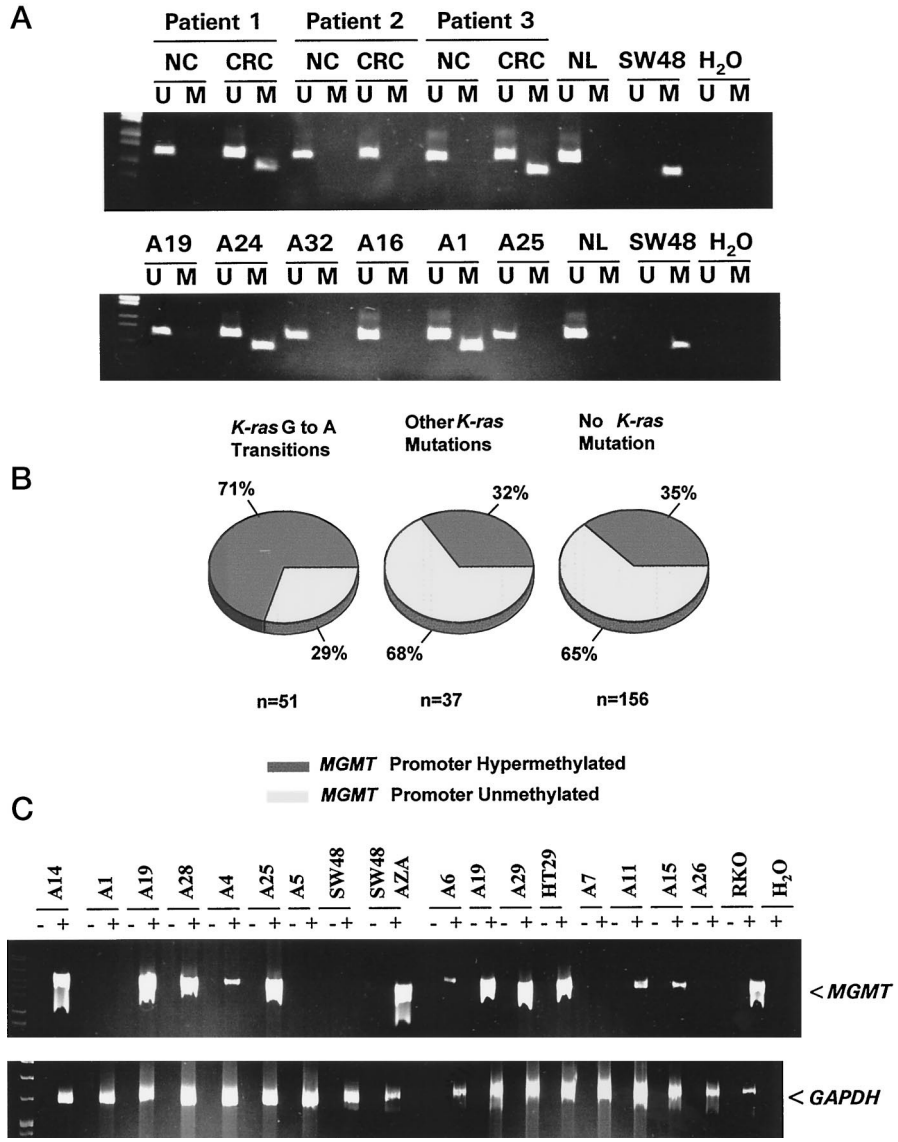

Fig. 1. *A*, examples of altered methylation status of the *MGMT* promoter in colorectal tumors and adenomas by methylation-specific PCR. The presence of a visible PCR product in *Lane U* indicates the presence of unmethylated genes of *MGMT*; the presence of product in *Lane M* indicates the presence of methylated genes of *MGMT*. *Top panel*, pairs of normal colon (*NC*) and primary colorectal carcinomas (*CRC*) from three patients. Note the presence of a product for hypermethylated *MGMT* sequences in patients 1 and 3. *Bottom panel*, primary colorectal adenomas (*A19*, and so forth). Note hypermethylation of *MGMT* in samples A24 and A1. Normal lymphocytes and the colorectal carcinoma cell line SW48 were used as negative and positive control for *MGMT* methylation, respectively. *B*, graphical distribution of *MGMT* hypermethylation-associated inactivation in colorectal tumorigenesis as a function of *K-ras* mutational spectrum. *C*, expression of *MGMT* determined by RT-PCR in colorectal adenomas and control colon cancer cell lines. + and −, the addition or absence of reverse transcriptase in the generation of cDNA. Expression of *MGMT* was abundant in the cell lines HT29 and RKO, unmethylated at *MGMT*. In the colorectal cell line SW48 methylated at MGMT, the expression was abolished and only was present after treatment with the demethylating agent 5-aza-2′-deoxycytidine. Adenomas without methylation also demonstrated strong *MGMT* expression (*A14*, *A19*, *A28*, *A25*, *A29*) whereas those with *MGMT* methylation had absent (*A1*, *A5*, *A7*, *A26*) or markedly reduced (*A4*, *A6*, *A11*, *A15*) levels of *MGMT* expression. The latter may reflect either normal tissue contamination or low levels of *MGMT* expression in the adenomatous cells themselves. *GAPDH* expression (*bottom panel*) demonstrates the integrity of the cDNA.

Discussion

A large amount of information concerning the genetic alterations found in human cancer has been compiled in recent years. However, we still know very little about the causes behind the DNA instability and enhanced mutational rates of a cancer cell. For the tumor suppressor *p53*, the deamination of CpG dinucleotides has been established as one of its important mutagenic events (8). Other genes with potential relevant roles in cancer, such as *BAX* or *TGFBRII*, suffer very specific mutations in sporadic tumors with compromised mismatch repair (21–22). Silencing of the DNA repair gene *hMLH1* by promoter hypermethylation is the probable event behind these defects in colorectal and endometrial cancer (23–25). In this report, we provide another example of the interaction between epigenetic and genetic events in human cancer. Our data suggest that epigenetic silencing of the DNA repair gene *MGMT* by hypermethylation is strongly associated with, and precedes, G to A mutations in *K-ras* in colorectal tumorigenesis. This change would result from persistence of the promutagenic adducts in the O^6 position of guanine that are not repaired by *MGMT*. Alkylating agents causing the promutagenic lesion may be provided from dietary nitrates reduced in the proximal colon by bacteria, by nitrosation of amines and amides derived of protein catabolism (26–28). A previous study had suggested that loss of enzyme activity in the normal mucosa was found in patients with G to A mutations in *K-ras* but could not make this association in tumors (29). However, in our study, the temporal relationship between inactivation of *MGMT* and the appearance of *K-ras* mutation in tumors with *MGMT* inactivation suggests a strong link between these two events.

It is of interest to take note of the tumor types for which *MGMT* promoter hypermethylation is a frequent event. It is known that in mouse models, the mammary tumors induced by MNU harbor characteristically G to A mutations in *H-ras* (30). Strikingly, the absence of *K-ras* mutations is a common feature of sporadic human breast carcinomas. Concordant with these phenomena, *MGMT* is not inactivated by promoter hypermethylation in breast tumors (12). On the other hand, for example, colorectal and non-small cell lung carcinomas have both changes: *K-ras* mutations (1, 2) and *MGMT* promoter hypermethylation (12). An interesting case arises from the brain tumors. Among the glial tumors, *MGMT* epigenetic inactivation (12) and *MGMT* loss or reduced activity (31, 32) are common features, but *ras* mutations are infrequent. These data may reflect a different spectrum of carcinogen exposure, *i.e.*, O^6-ethylguanine is removed

faster than the O^6-methylguanine that prevents *ras* mutations (33), or a different target gene more important for the biology of brain tumors than *K-ras* mutations. Thus, whereas the present study comments solely on G to A transitions in the *K-ras* gene, one important aspect of our data concerns the fact that each guanine in the human genome may generate a promutagenic lesion in the absence of correct repair. Other genetic alterations and mutations in target genes in human cancer that might be induced by *MGMT* epigenetic inactivation should receive close attention, and a search for these changes should be the focus of future investigations.

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