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Advances in Brief

Inactivation of the DNA Repair Gene O^6-*Methylguanine-DNA Methyltransferase* by Promoter Hypermethylation is a Common Event in Primary Human Neoplasia[1]

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Abstract

The DNA repair protein O^6-methylguanine DNA methyltransferase (MGMT) removes alkyl adducts from the O^6 position of guanine. MGMT expression is decreased in some tumor tissues, and lack of activity has been observed in some cell lines. Loss of expression is rarely due to deletion, mutation, or rearrangement of the *MGMT* gene, but methylation of discrete regions of the CpG island of *MGMT* has been associated with the silencing of the gene in cell lines. We used methylation-specific PCR to study the promoter methylation of the *MGMT* gene. All normal tissues and expressing cancer cell lines were unmethylated, whereas nonexpressing cancer cell lines were methylated. Among the more than 500 primary human tumors examined, *MGMT* hypermethylation was present in a subset of specific types of cancer. In gliomas and colorectal carcinomas, aberrant methylation was detected in 40% of the tumors, whereas in non-small cell lung carcinomas, lymphomas, and head and neck carcinomas, this alteration was found in 25% of the tumors. *MGMT* methylation was found rarely or not at all in other tumor types. We also analyzed MGMT expression by immunohistochemistry in relation to the methylation status in 31 primary tumors. The presence of aberrant hypermethylation was associated with loss of MGMT protein, in contrast to retention of protein in the majority of tumors without aberrant hypermethylation. Our results suggest that epigenetic inactivation of *MGMT* plays an important role in primary human neoplasia.

Introduction

MGMT[3] is a DNA repair protein that removes mutagenic and cytotoxic adducts from O^6-guanine in DNA (1). Alkylation of DNA at the O^6 position of guanine is an important step in the formation of mutations in cancer, primarily due to the tendency of the O^6-methylguanine to pair with thymine during replication, resulting in a conversion of guanine-cytosine to adenine-thymine pairs in DNA (2). Furthermore, the O^6-alkylguanine-DNA adduct may cross-link with the opposite cytosine residues, blocking DNA replication (3). *MGMT* protect cells against these lesions, transferring the alkyl group from the O^6-guanine in DNA to an active cysteine within its own sequence in a reaction that inactivates one *MGMT* molecule for each lesion repaired (1). Thus, the ability of a cell to withstand such damage is directly related to the number of *MGMT* molecules it contains and to the rate of *de novo* synthesis of *MGMT*.

The amounts of MGMT protein differ according to cellular type (4, 5) and are decreased in some tumors, with respect to their normal tissue counterpart (5, 6). A subset of tumor cell lines, termed Mer-, lack *MGMT* activity (7). Because loss of expression is not commonly due to deletion, mutation or rearrangement of the *MGMT* gene (7–9), or mRNA instability (10), other causes for loss of activity may be involved. Hypermethylation of normally unmethylated CpG islands in the promoter regions of many genes correlates with loss of transcription (11), and the human *MGMT* gene possesses a CpG island (12). Recent work has reported that methylation of discrete regions of the *MGMT* CpG island is associated with the silencing of the gene in cell lines (13–15). To study the relevance of the promoter hypermethylation of the *MGMT* gene *in vivo*, we have examined a series of more than 500 primary human tumors and corresponding normal tissues for *MGMT* aberrant methylation using MSP. Our results indicate that, in certain human cancers, *MGMT* function is lost frequently in association with hypermethylation of the promoter region and that this event may be an important step in human tumorigenesis.

Materials and Methods

MSP. DNA methylation patterns in the CpG island of the *MGMT* gene were determined by chemical modification of unmethylated, but not the methylated, cytosines to uracil and subsequent PCR using primers specific for either methylated or the modified unmethylated DNA (16). Primer sequences of *MGMT* were for the unmethylated reaction 5′-TTTGTGTTTTGATGTTT-GTAGGTTTTTGT-3′ (upper primer) and 5′-AACTCCACACTCTTCCAA-AAACAAAACA-3′ (lower primer) and for the methylated reaction 5′-TTT-CGACGTTCGTAGGTTTTCGC-3′ (upper primer) and 5′-GCACTCTTCC-GAAAACGAAACG-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 PCRs. Each PCR reaction (10 μl) was directly loaded onto nondenaturing 6% polyacrylamide gels, stained with ethidium bromide, and visualized under UV illumination.

Immunohistochemical Staining for MGMT. Sections of formalin-fixed, paraffin-embedded tissue (6 μm thick) were deparaffinized with xylenes for 30 min and dehydrated by using graded ethanols. Immunoperoxidase staining using diaminobenzidine as chromogen was performed with the TechMate 1000 automatic staining system (Ventana, BioTek Solutions, Tucson, AZ). Commercially available Mouse anti-*MGMT* monoclonal antibody (catalogue N99200; Novus Molecular Inc., San Diego, CA), previously referred to as mT3.1 (17), at 1:100 dilution was used. This antibody has previously been demonstrated to be useful for immunohistochemistry and to correlate with O^6-alkylguanine-DNA alkyltransferase activity (18). Nuclear staining was determined by two authors (S. R. H. and P. C. B.) who did not have knowledge of the molecular analysis of those samples.

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[3] The abbreviations used are: MGMT, O^6-methylguanine-DNA methyltransferase; CNU, chloroethylnitrosourea; Mer, methyl excision repair; MNU, *N*-methyl-*N*-nitrosourea; MSP, methylation-specific PCR; NSCLC, non-small cell lung carcinoma.

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Results

MGMT Promoter Hypermethylation. DNA obtained from 524 primary human tumors, cancer cell lines, and normal tissues was subjected to *MGMT* promoter methylation study using MSP. The region chosen for *MGMT* spans the area of greatest CpG density immediately 5′ to the transcription start site, in an area previously found to be hypermethylated in Mer- cell lines (14, 15). To determine the specificity of our approach, the colon carcinoma cell lines SW48 and HT29, reported previously as a Mer-deficient (Mer-) line and proficient (Mer+) line (19), respectively, were first studied. The SW48 cell line, which did not express *MGMT*, was fully methylated in this region, whereas the *MGMT*-expressing cell line HT29 was completely unmethylated (Fig. 1*B*). The *MGMT* promoter methylation status was investigated further in a set of normal human tissues, including brain, colonic mucosa, breast, pancreas, lung, and peripheral blood lymphocytes. Among these normal tissues tested, all were completely unmethylated at the *MGMT* gene (Fig. 1*A*). Thus, the methylation of *MGMT* identified in cell lines must have originated during tumor progression or in cell culture.

To address the relevance of the promoter hypermethylation of the *MGMT* gene *in vivo*, we examined a large series of primary human tumors for aberrant *MGMT* methylation using MSP. The results for the primary tumors with *MGMT* promoter hypermethylation are summarized in Table 1, and examples are shown in Fig. 1. Hypermethylation of MGMT was detected in many tumors. Aberrant *MGMT* hypermethylation was most frequent in primary gliomas and colorectal carcinomas, showing aberrant methylation in ~40% of the cases. Among the gliomas and colorectal carcinomas tested, 54 of 140 (38%) and 14 of 36 (38%), respectively, had *MGMT* promoter hypermethylation. Aberrant methylation of *MGMT* in gliomas was found in similar frequencies across the spectrum of grades: astrocytoma/oligodendroglioma (grade II) had hypermethylation in 8 of 26 (31%), anaplastic astrocytoma (grade III) in 10 of 20 (50%), and glioblastoma multiforme (grade IV) in 36 of 87 (41%). In contrast to the subtypes described above, the promoter of *MGMT* was unmethylated in meningiomas ($n = 25$), pilocytic astrocytomas ($n = 4$) and ependymomas ($n = 3$). A single medulloblastoma examined was hypermethylated at *MGMT*. We also examined cell lines of these tumor types and generally found a similar incidence of *MGMT* promoter methylation as seen in primary tumors, with two of four (50%) gliomas and three of seven (40%) colon cancer cell lines having aberrant methylation at the *MGMT* CpG island.

Table 1 *Hypermethylation of MGMT promoter in primary tumors*

	Primary tumors
Brain tumors	55/166 (33%)
Gliomas	54/140 (38%)
Nongliomas	1/26 (3%)
Colon cancer	14/36 (38%)
Lung cancer	10/41 (24%)
NSCLC	10/34 (29%)
SCLC	0/7
Head and neck carcinoma	6/21 (28%)
Lymphomas	15/61 (25%)
Breast cancer	0/36
Ovarian cancer	0/23
Endometrial cancer	0/17
Leukemias	2/31 (6%)
Pancreatic carcinoma	2/18 (11%)
Melanoma	2/18 (11%)
Renal carcinoma	1/12 (8%)
Bladder carcinoma	2/44 (4%)

A second group of neoplasms with frequent *MGMT* promoter methylation included lymphomas, NSCLCs, and head and neck carcinomas, where this alteration was found in ~25% of the cases (Table 1). In the non-Hodgkin's lymphomas studied, 15 of 62 (24%) were hypermethylated at *MGMT*. The presence of aberrant promoter methylation was a common feature in aggressive/high-grade lymphomas

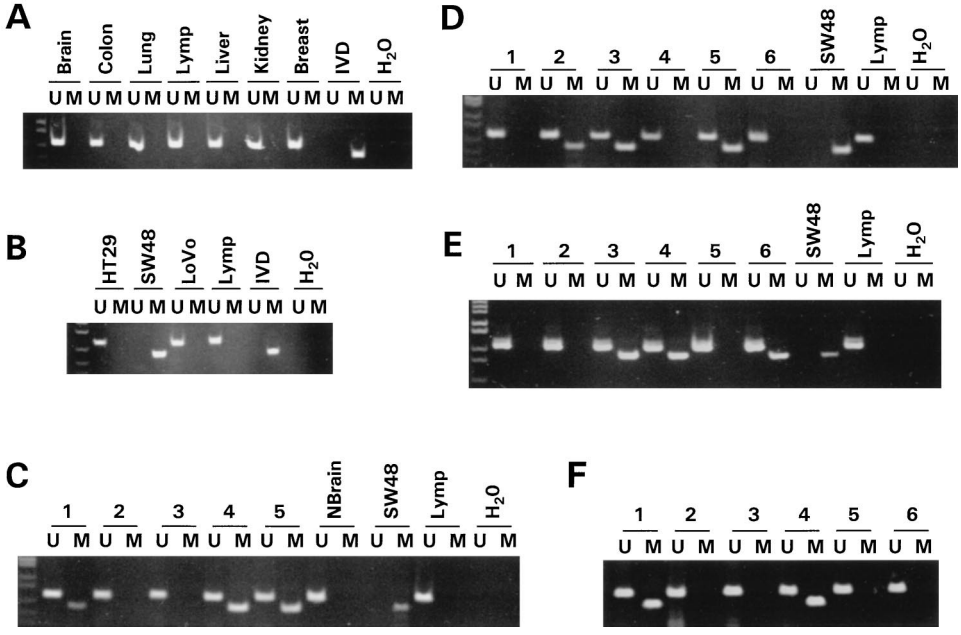

Fig. 1. MSP of *MGMT*. PBR32/Msp digest are shown at left as molecular weight markers. The presence of a visible PCR product in *Lane U* indicates the presence of unmethylated genes of *MGMT*; the presence of product in *Lane M* indicates the presence of methylated genes. A, normal tissues. Corresponding *Lanes* are: normal brain, colonic mucosa, lung, lymphocytes, liver, kidney, and breast; *in vitro* methylated DNA (*IVD*) as positive control for methylation; and water control for PCR. *B*, MSP of *MGMT* in cancer cell lines. Corresponding *Lanes* are: colon carcinoma cell lines HT-29 (Mer+), SW48 (Mer-), and LoVo (Mer+); *in vitro* methylated DNA as positive control for methylation; and water control for PCR. *C*, MSP of *MGMT* in primary gliomas. Corresponding *Lanes* are: primary gliomas (*1–5*), normal brain (*NBrain*), SW48 colon cancer cell line as positive control for methylation, normal lymphocytes as negative control for methylation, and water control for PCR reaction. *D*, MSP of MGMT in primary colorectal carcinomas. Corresponding *Lanes* are: primary colorectal carcinomas (*1–6*), *SW48* as positive control for methylation, normal lymphocytes as negative control for methylation, and water control for PCR reaction. *E*, MSP of *MGMT* in primary lung carcinomas. Corresponding *Lanes* are: primary lung carcinomas (*1–6*), SW48 as positive control for methylation, normal lymphocytes as negative control for methylation, and water control for PCR reaction. *F*, MSP of *MGMT* in primary lymphomas. Corresponding *Lanes* are primary lymphomas (*1–6*).

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Table 2 *Association of MGMT promoter hypermethylation and loss of MGMT expression in human primary tumors*

	Total	Hypermethylated	Unmethylated
All types (n = 31)			
Expressing tumors	18	1	17
Tumors with loss of expression	13	12	1
By types			
Brain tumors (n = 8)			
Expressing tumors	5		5
Tumors with loss of expression	3	3	
Lymphomas (n = 14)			
Expressing tumors	7		7
Tumors with loss of expression	7	6	1
Colorectal carcinomas (n = 9)			
Expressing tumors	6	1	5
Tumors with loss of expression	3	3	

where 11 of 14 (79%) were hypermethylated, including all 9 diffuse large cell lymphomas analyzed. In contrast, this *MGMT* epigenetic alteration was uncommon in indolent/low-grade lymphomas, where only 2 of 22 (9%) cases had *MGMT* methylation.

Hypermethylation of the *MGMT* promoter was not a universal finding in cancers in general, however. In contrast to the tumor types described above, other tumor types had infrequent *MGMT* promoter methylation: 2 of 18 (11%) pancreatic carcinomas, 2 of 18 (11%) melanomas, 1 of 12 (8%) renal carcinomas, 2 of 31 (6%) acute leukemias (1 AML and 1 ALL), and 2 of 44 (4%) bladder carcinomas. Finally, no carcinomas of the breast (n = 36), endometrium (n = 17), ovary (n = 23), or SCLCs (n = 7) analyzed had aberrant *MGMT* methylation.

In primary tumors, unlike cancer cell lines that were often either completely methylated or completely unmethylated in this region of the *MGMT* CpG island (Fig. 1*B*), hypermethylation of *MGMT* was always accompanied by amplification in the unmethylated reaction as well. The presence of this unmethylated *MGMT* region could indicate the presence of normal tissues in these nonmicrodissected samples, with unmethylated *MGMT* alleles, as observed in Fig. 1*A*. However, heterogeneity in the patterns of methylation in the tumor itself might also be present. To address this issue, we sought to determine the consequences of hypermethylation of the CpG island of *MGMT* on expression of this enzyme.

Expression of MGMT in Primary Tumors. Levels of MGMT expression often vary in primary tumors (5, 6). MGMT activity in primary tumors may be affected by contamination of tumor tissue with normal cells, such as endothelial cells, reactive astrocytes, or infiltrating lymphocytes, which express MGMT. Previous studies comparing MGMT activity, Western blot analysis, and immunohistochemistry have demonstrated only a moderate association between these methods of determining MGMT expression in colon carcinoma and normal colon (20). For these reasons, we analyzed MGMT expression by immunohistochemistry so that the expression in the neoplastic cells could be directly measured, using a previously characterized antibody (18). We first examined cell lines with known MGMT expression and methylation patterns to validate this approach. Expression of *MGMT* was absent in SW48 (Mer- and hypermethylated at the MGMT promoter), but was present in the Mer+ colon cancer cell lines HT29 and LoVo (data not shown).

Paraffin sections from 31 human primary tumors (including 8 brain tumors, 9 colon carcinomas, and 14 lymphomas) were then studied for MGMT expression. The immunohistochemical data related to *MGMT* promoter hypermethylation are shown in Table 2, and examples of the results obtained are shown in Fig. 2. All sections examined had nuclear staining of MGMT in normal cells adjacent to or within tumors, although in some cases this staining was weak. This provided an internal positive control. Among the 31 total tumors analyzed, 13 were judged to have loss of MGMT expression, whereas 18 tumors expressed MGMT. The level of expression observed in tumors varied greatly, and tumors that expressed MGMT often demonstrated higher expression than adjacent normal tissue. In the majority of these high MGMT expressing tumors, the expression was homogeneously increased. However, some cell to cell variation within in the neoplastic cells was observed. This finding is consistent with previous studies in these and other tissue types that tumor formation may be associated with increased levels of MGMT (20–23). However, a subset of tumors had diminished or absent MGMT expression, and in these tumors, protein was absent in the majority of tumor cells, with only occasional cells expressing detectable MGMT protein. The loss of staining was most uniform in the lymphomas, with greater cellular heterogeneity in the colon and brain tumors. Similar heterogeneity has been reported in colon (20) and lung (23) carcinomas demonstrating decreased expression of MGMT measured by immunohistochemistry.

When the expression data were matched to previous studies of the methylation of *MGMT*, 12 of 13 tumors that lack MGMT expression (92%) showed *MGMT* promoter hypermethylation, whereas 17 of 18 (94%) tumors that retained expression of MGMT were unmethylated at the *MGMT* CpG island (Fisher's exact test, <0.0001). This association between loss of MGMT expression and promoter hypermethylation was seen in each of the tumor types examined (Table 2). Among eight gliomas (five glioblastomas multiforme, two anaplastic astrocytomas, and one pilocytic astrocytoma), three had loss of protein expression and *MGMT* promoter hypermethylation, whereas five retained MGMT expression and were unmethylated at *MGMT*. Among nine primary colorectal carcinomas, three had lack of MGMT expression associated with abnormal *MGMT* methylation, whereas six of seven (85%) expressing tumors were unmethylated at *MGMT*. In all cases, adjacent normal colon epithelia expressed the MGMT protein. In two colonic carcinoma cases, we were able to examine MGMT expression in both primary and metastatic lesions. A hepatic metastasis from a primary tumor hypermethylated at *MGMT*, which lacked expression of the protein, also demonstrated loss of MGMT expression, whereas a lymph node metastasis from an unmethylated primary tumor, which expressed MGMT, retained MGMT expression in the metastasis. Finally, the analysis of 14 lymphomas (8 diffuse lymphomas, 5 follicular lymphomas, and 1 marginal zone lymphoma) revealed that among seven tumors showing loss of MGMT expression, six had *MGMT* promoter methylation, whereas all seven expressing tumors were unmethylated at the *MGMT* loci.

Discussion

In cell culture, a causal relationship between methylation of the *MGMT* CpG island and transcriptional silencing of the gene has been demonstrated (13–15). Our data now demonstrate that *MGMT* promoter hypermethylation associated with loss of expression is frequent in primary human neoplasia as well. The tight correlation between *MGMT* CpG island methylation and loss of expression in these tumors provides an explanation for the loss of MGMT activity previously reported in a subset of many tumor types (5, 6, 20, 21, 23, 24). However, in tumor types where we did not detect *MGMT* hypermethylation, such as breast, ovarian carcinoma, or meningioma, decreased *MGMT* activity is infrequent (5, 6, 24).

Because *MGMT* plays a major role in the repair of the O^6-methylguanine DNA adducts, which are formed after exposure to methylating agents [including the monofunctional alkylating agents MNU or MNNG (*N*-methyl-*N*'-nitro-*N*-nitrosoguanidine)] and to clinical drugs such as dacarbazine, procarbazine, temozolomide, lomustine [CCNU, 1-(2-chloroethyl)-3-cyclohesyl-1-nitrosourea], nimustine [ACNU, 1-(4-amino-2-methyl-5-pyrimidinyl)methyl-3–2(2-chlo-

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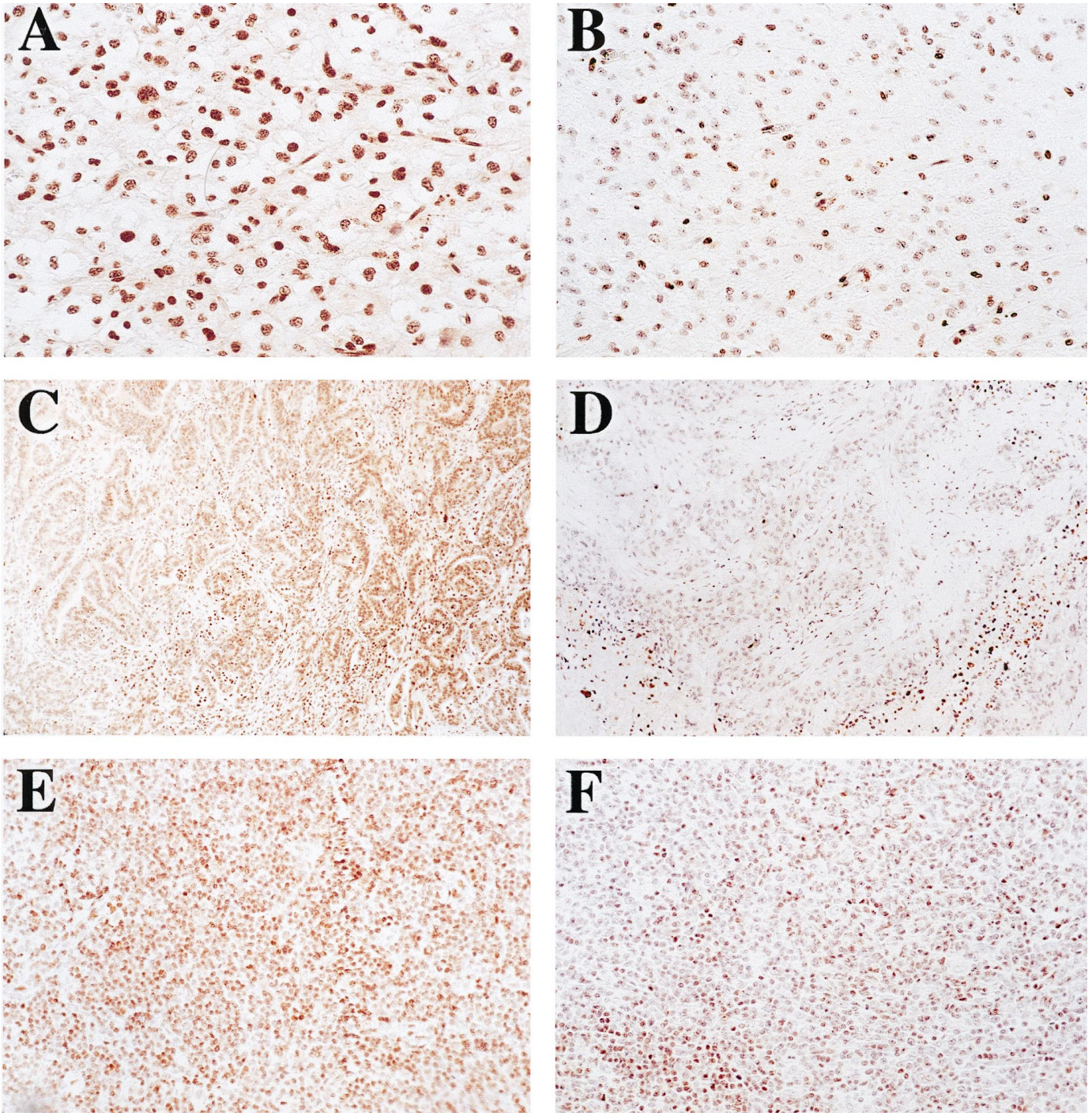

Fig. 2. Immunohistochemistry of *MGMT* gene product in primary human tumors. *A*, anaplastic astrocytoma unmethylated at *MGMT* showing expression of the protein in tumor cell nuclei. *B*, glioblastoma multiforme with *MGMT* promoter hypermethylation lacking expression in the majority of malignant cells. *C*, colonic carcinoma unmethylated at *MGMT* expressing the protein, whereas another colonic carcinoma in *D* with aberrant methylation at *MGMT* shows complete lack of expression in tumor nuclei, but expression in stromal cells. *E*, follicular lymphoma unmethylated at *MGMT* promoter showing expression of the protein. *F*, diffuse lymphoma hypermethylated at *MGMT* demonstrating loss of *MGMT* expression.

roethyl)-3-nitrousourea], and carmustine [BCNU, 1,3-bis(2-chloroethyl)-1-nitrousourea], our results identify potentially critical events in carcinogenesis and may define a set of chemosensitive tumors. During DNA replication, O^6-methylguanine can pair with thymine, resulting in conversion of guanine-cytosine to adenine-thymine pairs in DNA (2). Such mutations are often present in tumors induced by alkylating agents (25). To counteract these effects, *MGMT* protein can remove such adducts before mutations result. Thus, *MGMT* gene knockout mice have a higher susceptibility to MNU-induced tumorigenesis, specifically manifesting more frequent lung adenomas and thymic lymphomas when compared with wild-type mice (26). Our findings that primary NSCLCs and lymphomas are tumor types with frequent methylation-associated inactivation of the *MGMT* gene suggest that this epigenetic alteration may confer an increased risk of alkylating agent-induced carcinogenesis in the systems of these organs.

The importance of *MGMT* in carcinogenesis is also evident in the *MGMT* transgenic mice, who possess protection against skin carcino-

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genesis, thymic lymphomas, and aberrant crypt foci of the colon induced by alkylating agents (27–29). Interestingly, simple methylating agents, such as MNU, ACNU (a CNU derivative), and NNK [4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone], a nitrosamine present in tobacco and tobacco smoke, have been shown to induce *ras* mutations (30–32). Tumor types with silencing of the *MGMT* by aberrant promoter hypermethylation in this study include those that have a frequent rate of K-*ras* mutations described in the literature, such as colon, lung, and head and neck carcinomas (30). This suggests that one potential consequence of loss of MGMT expression could be an increase in susceptibility to K-*ras* mutation. The MGMT transgenic mouse, in fact, is not only protected against aberrant crypt foci formation, but also has a lower incidence of crypt foci with G to A mutations in K-*ras* (29). However, other tumors associated with carcinogen exposure, such as bladder carcinoma, and other tumors with a high rate of K-*ras* mutations, such as pancreatic carcinoma, were not frequent targets of *MGMT* promoter hypermethylation, suggesting that inactivation of MGMT is not required for the acquisition of K-*ras* mutations.

From a clinical standpoint, *MGMT* promoter hypermethylation with subsequent loss of MGMT expression might play a role in modulating chemosensitivity to alkylating agents. CNUs and methylating agents, such as streptozotocin, are commonly used in the treatment of neoplastic diseases such as brain tumors, colon carcinoma, malignant melanoma, Hodgkin's disease, lymphoma, myeloma, and skin cancer. Among the tumors studied in the present study, gliomas, colon carcinomas, and lymphomas had the highest frequency of *MGMT* promoter hypermethylation. These tumor types, in addition to NSCLC, have all been reported to have decreased *MGMT* expression and activity (5, 6, 20, 21, 23, 24, 33) in a subset of tumors. Thus, the decreased *MGMT* activity observed in a significant fraction of these tumors and the response of the former tumor types to CNUs may be due, in part, to the lack of O^6-chloroethylguanine repair by inactivation at *MGMT* by aberrant promoter methylation. The tight correlation between loss of expression at the protein level and hypermethylation of the *MGMT* 5′ CpG island we report in our study directly links these two processes *in vivo* and suggests an important role for aberrant promoter hypermethylation of *MGMT* in primary human cancer.

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Inactivation of the DNA Repair Gene O^6-*Methylguanine-DNA Methyltransferase* by Promoter Hypermethylation is a Common Event in Primary Human Neoplasia

Manel Esteller, Stanley R. Hamilton, Peter C. Burger, et al.

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