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Advances in Brief

Promoter Hypermethylation of the DNA Repair Gene *O*^{6}-*Methylguanine-DNA Methyltransferase* Is Associated with the Presence of G:C to A:T Transition Mutations in *p53* in Human Colorectal Tumorigenesis[1]

Manel Esteller, Rosa-Ana Risques, Minoru Toyota, Gabriel Capella, Victor Moreno, Miquel Angel Peinado, Stephen B. Baylin, and James G. Herman[2]

Tumor Biology, The Johns Hopkins Oncology Center, Baltimore, Maryland 21231 [M. E., S. B. B., J. G. H.]; Cancer Epigenetics Laboratory, Molecular Pathology Program, Centro Nacional de Investigaciones Oncologicas, Majadahonda 28220, Spain [M. E.]; First Department of Internal Medicine, Sapporo Medical University, Sapporo 060-8543, Japan [M. T.]; Institut Catala d'Oncologia and Institut de Recerca Oncologica, Hospital Duran i Reynals, Barcelona, Catalonia 08907, Spain [R-A. R., G. C., V. M., M. A. P.]

Abstract

Defects in DNA repair may be responsible for the genesis of mutations in key genes in cancer cells. The tumor suppressor gene *p53* is commonly mutated in human cancer by missense point mutations, most of them G:C to A:T transitions. A recognized cause for this type of change is spontaneous deamination of the methylcytosine. However, the persistence of a premutagenic O^6-methylguanine can also be invoked. This last lesion is removed in the normal cell by the DNA repair enzyme O^6-methylguanine-DNA methyltransferase (*MGMT*). In many tumor types, epigenetic silencing of *MGMT* by promoter hypermethylation has been demonstrated and linked to the appearance of G to A mutations in the *K-ras* oncogene in colorectal tumors. To study the relevance of defective *MGMT* function by aberrant methylation in relation to the presence of *p53* mutations, we studied 314 colorectal tumors for *MGMT* promoter hypermethylation and *p53* mutational spectrum. Inactivation of *MGMT* by aberrant methylation was associated with the appearance of G:C to A:T transition mutations at *p53* (Fischer's exact test, two-tailed; $P = 0.01$). Overall, *MGMT* methylated tumors displayed *p53* transition mutations in 43 of 126 (34%) cases, whereas *MGMT* unmethylated tumors only showed G:C to A:T changes in 37 of 188 (19%) tumors. A more striking association was found in G:C to A:T transitions in non-CpG dinucleotides; 71% (12 of 17) of the total non-CpG transition mutations in *p53* were observed in *MGMT* aberrantly methylated tumors (Fischer's exact test, two-tailed; $P = 0.008$). Our data suggest that epigenetic silencing of *MGMT* by promoter hypermethylation may lead to G:C to A:T transition mutations in *p53*.

Introduction

The mutational spectrum of oncogenes and tumor suppressor genes can reflect specific causes (1). Exogenous and endogenous compounds are known to cause DNA damage (2), including deletions, insertions, and base substitutions, either transversions (change of purine to pyrimidine or *vice versa*) or transitions (change or purine to another purine or pyrimidine to another pyrimidine; Ref. 3). Well-known sources of the spontaneous generation of point mutations include: deamination of cytosine and 5-methylcytosine to uracil and thymine, respectively; depurination; DNA polymerase infidelity; and oxidative damage from endogenously produced free radicals (1, 3).

Abnormalities in DNA repair and replication have long been considered as key elements in the genesis of mutations. Several mechanisms can be invoked to contribute to the infidelity of DNA synthesis, including imbalances in deoxynucleotide triphosphate pools, mutations in DNA polymerase-α, and slippage of DNA polymerase at nucleotide repeats (4–6). However, the relevance of each mechanism and candidate gene is still unknown. Recently, epigenetic alterations in two DNA repair genes, the mismatch repair gene *hMLH1* and *MGMT*,[3] have been linked to very specific genetic mutations in sporadic tumors (7–11). Germ-line mutations in the two DNA mismatch repair genes *hMLH1* and *hMSH2* are the genetic abnormalities responsible for the vast majority of hereditary nonpolyposis colorectal carcinoma cases (reviewed in Ref. 12), where the presence of deletions and insertions in the microsatellite sequence is a common hallmark. However, in the nonfamilial cases, the presence of microsatellite instability is attributable to methylation-associated silencing of hMLH1 (7–11), which leads ultimately to mutations in target genes such as *Bax* or *TGFRβII* (reviewed in Ref. 13).

The case of *MGMT* is also particularly interesting. The persistence of O^6-methylguanine adducts, resulting from alkylating agents, may cause DNA polymerase to misread the base pairing because of the altered hydrogen-bonding properties of a base that contains an additional methyl or ethyl group. Thus, O^6-methylguanine is read as an adenine and mispairs with thymine (14). Supporting this data, the most common mutations caused by alkylating agents are G:C to A:T transitions (14), exemplified in the frequent generation of G to A transitions in the oncogene *K-ras* when the carcinogen *N*-methylnitrosourea (that forms O^6-methylguanine adducts) is used in experimentally induced tumor systems (15). Avoidance of the mutagenic effect is directly related to the presence of a functional MGMT protein (16). *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 show that endogenous *MGMT* expression protects mammalian cell lines from spontaneous G:C to A:T transitions in the *aprt* gene (17). Animal models also show that transgenic mice overexpressing *MGMT* are protected against O^6-methylguanine-DNA adducts caused by methylnitrosourea (18) and against G to A mutations in *K-ras* in aberrant colorectal crypt foci and lung tumors (19, 20). Furthermore, we have recently shown that *MGMT* is transcriptionally silenced by promoter hypermethylation in a wide spectrum of human neoplasms (21) and provided the first evidence in human primary tumors of the linkage between *MGMT* epigenetic inactivation and the appearance of G to A mutations in *K-ras* (11).

MGMT inactivation is not likely to be limited to an association with only *K-ras* mutations. The tumor suppressor *p53* is the most commonly mutated gene in human cancer, and transition mutations constitute the main type of *p53* mutations observed (22, 23). Approxi-

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[2] To whom requests for reprints should be addressed, at Tumor Biology, The Johns Hopkins Oncology Center, Room 543, 1650 Orleans Street, Baltimore, MD 21231. Phone: (410) 955-8506; Fax: (410) 614-9884; E-mail: hermanji@welchlink.welch.jhu.edu.

[3] The abbreviation used is: *MGMT*, O^6-methylguanine-DNA methyltransferase.

mately 52% of the mutational events are missense transitional changes, and, of this subset, ~72% are G:C to A:T transitions (22). The profile of the mutational spectrum varies according to the tumor type. Lung and head and neck tumors of smokers have a higher number of transversions, whereas colorectal tumors have the highest rate of transition mutations, reaching 70% of the total number of *p53* mutations (22). These last mutations occur frequently in CpG dinucleotides, which are normally methylated (23–25), through increased rates of spontaneous deamination at methylcytosine; although other mechanisms are also conceivable. However, 17% of *p53* mutations are transition mutations in non-CpG dinucleotides, where this causality cannot be invoked (22).

Thus, G:C to A:T changes in *p53* in non-CpG and CpG dinucleotides could be attributable, in part, to a defect in *MGMT* that allows the persistence of O^6-methylguanine and its reading as an adenine. To address this question, we have examined in a large collection of colorectal tumors ($n = 314$) whether the presence of *MGMT* epigenetic inactivation was linked to transition mutations in *p53*. Our data show that promoter hypermethylation of *MGMT* is strongly linked to the presence of G:C to A:T transition mutations in *p53*, particularly in non-CpG dinucleotides.

Materials and Methods

Tumor Samples. Initially, 235 colorectal carcinoma samples were collected from patients operated on at the Hospital de Sant Pau and the Hospital de Bellvitge in Barcelona, Catalonia, Spain, under the supervision of Drs. Gabriel Capella and Miquel Angel Peinado. The study protocol was approved by the ethics committee. One hundred and twenty of these samples were characterized previously for MGMT methylation (11). Subsequently, an additional 14 colorectal carcinomas and 65 colorectal adenomas were obtained from surgical resection specimens of patients operated on 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. All 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 *MGMT* were determined by chemical modification of the unmethylated, but not the methylated, cytosines to uracil and subsequent PCR using primers specific for either methylated or the modified unmethylated DNA (21, 26). Primer sequences of *MGMT* for the unmethylated reaction were 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); for the methylated reaction they were 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. Then DNA samples were 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.

Detection of *p53* Mutations. *p53* mutations in exons 4–9 were analyzed by single-strand conformational polymorphism analysis. Briefly, a first PCR was performed using primers 12979U (GCT GCC GTG TTC CAG TTG CT) and 14875D (AGG CAT CAC TGC CCC CTG AT). The resulting 1897-bp fragment was then used as a template to amplify separately a fragment of 410 bp including exons 5 and 6 (with primers 13054U, TAC TCC CCT GCC CTC AAC AAG; and 13463D, CTC CTC CCA GAG ACC CCA GT) and a fragment of 622 bp including exons 7 and 8 (with primers 13966U, CTGGC-CTCATCTTGGGCCTG; and 14587D, CTCGCTTAGTGCTCCCTGGG). These two fragments were then digested with the restriction enzyme *Hpa*II, and the resulting fragments were run on a 6% polyacrylamide gel without glycerol (0.2 h at 30 W and 5–6 h at 6 W) and with 10% glycerol (0.2 h at 30 W and 13–14 h at 6 W) to detect mobility shifts. Mutations were confirmed by direct cycle sequencing of the PCR products using the AmpliCycle Sequencing Kit (Perkin-Elmer, Branchburg, NJ). Exons 4 and 9 were only analyzed on those samples negative for mutations in exons 5–8. Exon 4 was amplified directly from DNA using primers 12019U (GTC CCC CTT GCC GTC CCA AG) and 12349D (TAC GGC CAG GCA TTG AAG TC). The resulting 331-bp fragment was run without previous digestion on a 6% polyacrilamide/10% glycerol gel for 0.2 h at 30 W and for 19 h at 6 W. To analyze exon 9, a fragment of 788 bp including exons 7–9 was amplified with primers 13966U (CTG GCC TCA TCT TGG GCC TG) and 14753D (CTG AAG GGT GAA ATA TTC TCC) and digested with *Hha*I to produce two fragments of 548 and 240 bp, the last one containing exon 9.

Results

***MGMT* Promoter Hypermethylation and the Occurrence and Type of *p53* Mutations.** Among 314 colorectal lesions, 249 carcinomas, and 65 adenomas, 126 (40%) demonstrated hypermethylation of *MGMT*. Representative examples of the methylation analysis in colorectal carcinomas and adenomas are shown in Fig. 1*A*. Of these 314 colorectal samples, 135 (43%) had mutant *p53*. The most common type of *p53* mutations were G:C to A:T transitions [81 of 135 (60%)], whereas transversion mutations and insertions/deletions were 54 of 135 (40%). The G:C to A:T transitions occurred in CpG dinucleotides in 64 of 81 (79%) cases and in non-CpG dinucleotides in 17 of 81 (21%) tumors.

A trend was observed between the presence of *MGMT* promoter hypermethylation and the presence of *p53* mutations, because 61 of 135 tumors (45%) with mutant *p53* had methylated *MGMT*, whereas only 65 of the 179 tumors (36%) without *p53* mutations had methylated *MGMT*. When the presence of aberrant methylation of *MGMT* was sorted out according to the molecular type of *p53* mutation, no differences in *MGMT* methylation were observed between the wild-type *p53* group and the *p53* mutants because of transversions or insertion/deletions. However, *MGMT* promoter hypermethylation was significantly associated with the presence of G:C to A:T transition mutations in *p53* (Fischer's exact test, two-tailed; $P = 0.01$). Overall, *MGMT* methylated tumors displayed *p53* transitions in 43 of 126 (34%) cases, whereas *MGMT* unmethylated tumors only showed G:C to A:T changes in 37 of 188 (20%) tumors. From the *p53* mutational standpoint, tumors with G:C to A:T transitions had *MGMT* methylation in 43 of 81 cases (53%) cases, whereas tumors with other type of *p53* mutations or wild-type sequence displayed *MGMT* methylation only in 83 of 223 (37%) cases. The clustering of *MGMT* methylation-associated inactivation with the existence of p53 transitions mutations was even more dramatic when these were subdivided according to their occurrence or not in a CpG dinucleotide: 71% (12 of 17) of the total non-CpG transition mutations in *p53* were observed in *MGMT* aberrantly methylated tumors. Thus, *MGMT* promoter hypermethylation was significantly linked to the appearance of G:C to A:T transition mutations in the non-CpG dinucleotides of *p53* when compared with the wild-type *p53* colorectal tumors (Fischer's exact test, two-tailed; $P = 0.008$) or the other types of *p53* mutations (Fischer's exact test; two-tailed; $P = 0.03$). Fig. 1*B* displays in a graphic way the distribution of MGMT promoter hypermethylation according to the type of *p53* mutational event.

Discussion

In recent years, cancer has begun to be understood not only as a genetic disease, but also as an epigenetic one (27, 28). The genetic and epigenetic pathways are not isolated, but rather there is a complex network of connections between both. For example, germ-line point

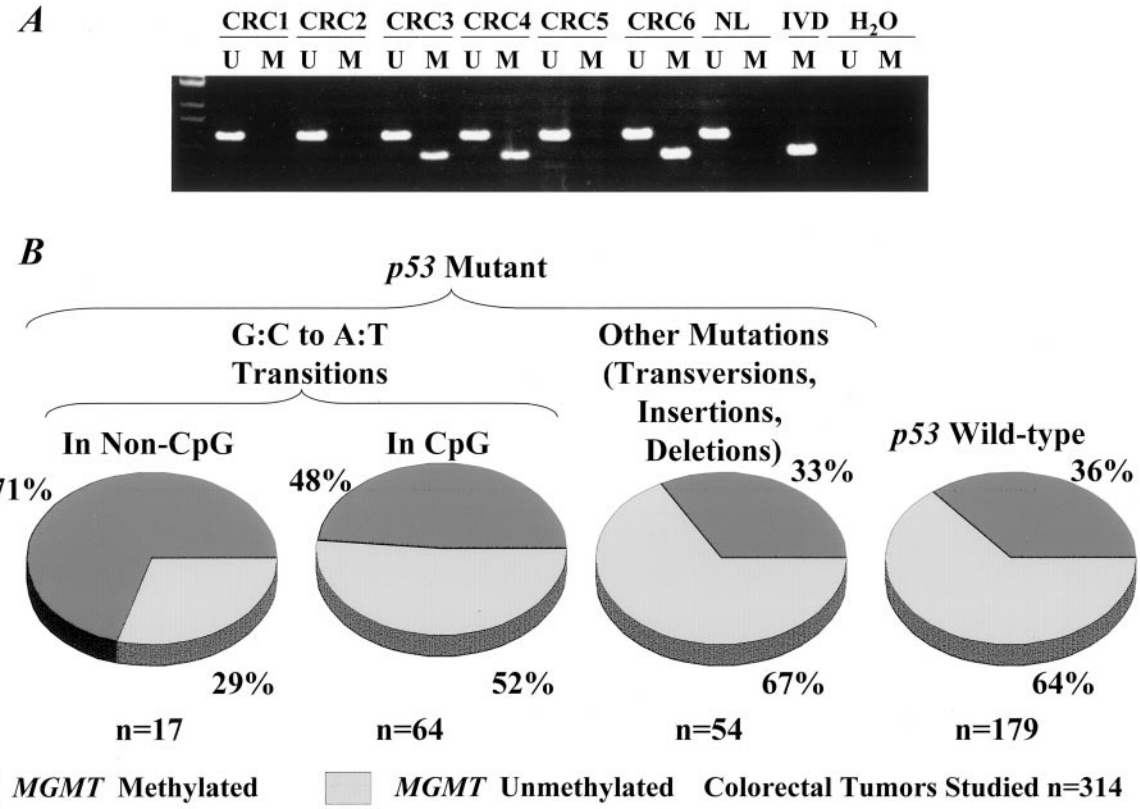

Fig. 1. *A*, examples of altered methylation status of the *MGMT* promoter in colorectal tumors by methylation-specific PCR. The presence of a visible PCR product in *Lane U* indicates the presence of unmethylated genes of *MGMT*; the presence of product in *Lane M* indicates the presence of methylated genes of *MGMT*. Note the presence of a product for hypermethylated *MGMT* sequences in patients CRC3, CRC4, and CRC6 (*CRC*, colorectal tumors). Normal lymphocytes (*NL*) and *in vitro* methylated DNA (*IVD*) were used as negative and positive controls for *MGMT* methylation, respectively. *B*, graphical distribution of *MGMT* hypermethylation-associated inactivation in colorectal tumorigenesis as a function of p53 mutational spectrum.

mutations in the DNA methyltransferase 3b affect methylation patterns of the genome (29), and promoter hypermethylation of the mismatch repair gene *hMLH1* in sporadic tumors causes the dramatic mutator phenotype known as microsatellite instability (7–10). The epigenetic silencing of the DNA repair *MGMT* is another example of how abnormal methylation may lead to increased rates of mutation.

Our finding that *MGMT* promoter hypermethylation is associated with the presence of G:C to A:T transition mutations in *p53* provides another target gene for *MGMT*-deficiency in human cancer cells, after the initial linkage between *MGMT* methylation and G to A mutations in *K-ras* (11). We have been able to observe a direct relation between *MGMT* aberrant methylation and G:C to A:T transitions in *p53* in colorectal tumors, even with the "masking" effect attributable to spontaneous deamination of the methylcytosine in the CpG dinucleotide. It is not known at the present moment which is the real quantitative contribution of each lesion, a promutagenic O^6-methylguanine or a methylcytosine, to the appearance of *p53* mutations. However, when the G:C to A:T mutation occurs in a non-CpG dinucleotide, the association with *MGMT* inactivation is clearly evident.

The relation observed with *p53* is itself highly relevant, because transition mutations of *p53* in human cancer are common and happen in all malignant cell types. Some of these tumors, such as those derived from the brain, lung, head and neck, and lymphomas, also have *MGMT* hypermethylation-associated inactivation (21). Thus, the epigenetic defect in the *MGMT* gene may be proposed as a clear predisposition factor to acquire *p53* transition mutations also in these cell types. In the case of colorectal tumors, the promutagenic lesion affecting the O^6-guanine can be caused by alkylating agents provided from dietary nitrates reduced in the proximal colon by bacteria or by nitrosation of amines and amides derived from protein catabolism (30–32). Interestingly, the appearance of gliomas, the second tumor type with a higher rate of *MGMT* methylation, also has been related to nitrosamine exposure (33), and the presence of *MGMT* promoter hypermethylation "marks" those tumors highly sensitive to chemotherapy (34). Additional evidence of causality between the epigenetic and genetic event is provided by the timing of both alterations. *MGMT* abnormal methylation can be found in early lesions, such as colorectal adenomas, preceding the appearance of *p53* mutations in the more advanced stages.

Another very interesting aspect, derived from our present research, that needs to be studied further in the future is to know how many target genes and sequences can be affected by the lack of *MGMT* repair capacity. Putative target genes where similar associations may be observed include other tumor suppressor genes or oncogenes such as *H-ras* and *N-ras*. It is even possible, using the similarity with the mismatch repair deficiency, that those tumors with *MGMT* promoter hypermethylation have a special mutator phenotype characterized for numerous transition mutations, some affecting important genes, others affecting only repeated sequences, through their genomes. Overall, our data strongly suggest that the silencing of the DNA repair gene *MGMT* by promoter hypermethylation confers to the cancer cell additional mutability, specifically the capacity to acquire G:C to A:T transition mutations. This finding provides us with another example, like hMLH1, of how epigenetic lesions can cause genetic lesions.

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