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

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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\textsuperscript{6}-methylguanine can also be invoked. This last lesion is removed in the normal cell by the DNA repair enzyme O\textsuperscript{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 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 inefficiency; 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-\(\alpha\), 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\textsuperscript{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 TGFB\textsubscript{3} (reviewed in Ref. 13).

The case of MGMT is also particularly interesting. The persistence of O\textsuperscript{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\textsuperscript{6}-methylguanine is read as an adenine and mismaps 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-methyl-\(N\)-nitrosourea (that forms O\textsuperscript{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 chloroethylnitrations, at the O\textsuperscript{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\textsuperscript{6}-methylguanine-DNA adducts caused by methylphenoxazone (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). Approximately

\textsuperscript{1}The abbreviation used is: MGMT, O\textsuperscript{6}-methylguanine-DNA methyltransferase.

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The presence of G:C to A:T transition mutations in non-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 O6-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 for MGMT or 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 CAC AAA CAA AAC A-3' (lower primer); for the methylated reaction they were 5'-TTT CCA CGT TCG TAG GTT TTC GC-3' (upper primer) and 5'-CA CTC TTC CTC 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 CTG 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 GAC ACC ACA CTA) and a fragment of 622 bp including exons 7 and 8 (with primers 13966U, CTGGCCCTCATTCTGGCCGCT; and 14587D, CTTCGTTAGTGCTCTCCTCGGG). These two fragments were then digested with the restriction enzyme HpaII, 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 GTG CCA AG) and 12349D (TAC GGC CAG GCA TTG AAG TC). The resulting 331-bp fragment was run without previous digestion on a 6% polyacrylamide/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 HpaII 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. 1A. 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 insertions/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. 1B 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
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 to A mutations in K-ras (11). We have been able to observe a direct relation between MGMT aberrant 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 to A mutations in K-ras (11). We have been able to observe a direct relation between MGMT aberrant methylation and G to A mutations in K-ras (11).

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.

References

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