Advances in Brief

O\textsuperscript{6}-Methylguanine-DNA Methyltransferase Promoter Hypermethylation Shifts the p53 Mutational Spectrum in Non-Small Cell Lung Cancer\textsuperscript{1}

Patrick Wolf, Ying Chuan Hu, Kara Doffek, David Sidransky, and Steven A. Ahrendt\textsuperscript{2}

Department of Surgery, Medical College of Wisconsin, Milwaukee, Wisconsin 53226 [P. W., K. D.]; Department of Otolaryngology-Head and Neck Surgery, The Johns Hopkins Medical Institutions, Baltimore, Maryland 21287 [D. S.]; and Department of Surgery, University of Rochester, Rochester, New York 14642 [Y. C. H., S. A. A.]

Abstract

The DNA repair protein O\textsubscript{6}-methylguanine-DNA methyltransferase (MGMT) removes mutagenic adducts from the O\textsubscript{6} position of guanine, thereby protecting the genome against G to A transition mutations. MGMT is inactivated by promoter hypermethylation in many human cancers and has been associated with G to A mutations in K-ras in colorectal cancer. We hypothesized that MGMT promoter hypermethylation would be associated with an increase in G to A transitions in the p53 gene in non-small cell lung cancer (NSCLC). p53 mutations were detected by both denaturing sequencing and p53 GeneChip analysis in 92 patients with primary NSCLC. Methylation of the promoter region of the MGMT gene was determined using methylation-specific PCR and was present in 27 of 92 (29%) tumors. Hypermethylation of the MGMT promoter was more common in adenocarcinoma than in other histological types of NSCLC and was also more common in poorly differentiated tumors. MGMT promoter hypermethylation was present significantly more often in tumors with a G to A mutation in p53 (9 of 14; 64%) than in tumors with other types of p53 mutations (11 of 41; 27%; P = 0.02) or in tumors with wild-type p53 (7 of 37; 18%; P = 0.006). MGMT promoter hypermethylation was also strongly associated with G to A transitions at CpG sites. Inactivation of the MGMT gene by promoter hypermethylation alters the pattern of p53 mutation in NSCLC.

Introduction

\textit{MGMT} is a DNA repair gene critical for the removal of mutagenic adducts from the O\textsubscript{6} position of guanine (1, 2). In the absence of MGMT activity, O\textsubscript{6}-alkylguanine mispairs with thymine during DNA replication and results in guanine-cytosine to adenine-thymine transitions (1, 3). MGMT prevents this conversion by transferring the alkyl group from the O\textsubscript{6} position of guanine to a cysteine residue within its own sequence in a stoichiometric reaction that permanently inactivates its acceptor site (1, 4). MGMT expression is quite variable among normal human tissues and is increased by exposure to cigarette smoke (5–7). MGMT activity is absent in several human neoplasms including colon and lung cancer, gliomas, and lymphoma (1, 5, 6, 8, 9). Mutations have not been identified within the \textit{MGMT} gene, and inactivation in human cancer is most likely attributable to hypermethylation of normally unmethylated CpG islands in the \textit{MGMT} promoter region (1, 10, 11). Although selective gene inactivation through promoter methylation plays a crucial role in several cellular events, including genomic imprinting and X-chromosome inactivation, methylation of tumor suppressor or DNA repair genes may also play a significant role in neoplastic progression (10, 12).

Most human cancers originate through the cumulative activation and inactivation of oncogenes and tumor suppressor genes, respectively. Epigenetic events, such as hypermethylation of CpG islands within the promoter region of many genes, may influence this process (10), e.g., silencing of the mismatch repair gene \textit{hMLH1} is associated with sporadic colorectal, gastric, and endometrial cancer (13–15). Defective mismatch repair subsequently leads to additional mutations in other genes, such as \textit{BAX} and \textit{TGFBRII} (16, 17). Early \textit{MGMT} inactivation has also been linked with gene mutations in other critical growth regulatory genes. K-ras mutations and especially G to A mutations are more common in colon cancer after \textit{MGMT} inactivation (18). However, K-ras mutations are less common in NSCLC than colorectal cancer, occur in only 20–40% of lung adenocarcinomas, and are rare in other histological types (19, 20). In NSCLC, tumor suppressor gene inactivation through point mutation is observed most frequently in p53. G to T transversions are the most common p53 mutation in lung cancer, and their presence is linked to tobacco use in lung and other smoking-associated neoplasms (21–23). Several of the p53 mutational hotspots in NSCLC (codons 157, 158, 245, 248, and 273) occur at methylated CpG sites, which are preferential binding sites for BPDE and other PAHs (24–26). In addition, repair of DNA adducts at these sites is also slower than repair at other sites (27). Although G to T transversions are the most common p53 mutation in NSCLC, G to A transitions also occur frequently and account for 35% of mutations at CpG sites and 18–24% of all p53 mutations (21, 23). Furthermore, G to A mutations occur more frequently in lung adenocarcinoma and in NSCLC from nonsmokers (22). Because G to A transitions are the consequence of \textit{MGMT} inactivation, we examined the relationship between \textit{MGMT} promoter hypermethylation and p53 mutational spectrum in NSCLC.

Materials and Methods

Sample Collection. Primary tumor was collected from 92 patients with NSCLC undergoing surgical resection at the Johns Hopkins Hospital or the Medical College of Wisconsin. Patients were selected from a larger group of samples analyzed previously for p53 gene mutations (21, 28) to include all of the G to A transitions (n = 14) and a randomly chosen representative group of other p53 mutations (n = 41) and wild-type tumors (n = 37). None of the patients received preoperative chemotherapy. Pathological stage was determined using the revised International System for Staging Lung Cancer (29). Both the Joint Committee on Clinical Investigation of The Johns Hopkins School of Medicine and the Institutional Review Board of the Medical College of Wisconsin approved this research protocol. Written informed consent was obtained from all patients.

Tumor samples were promptly frozen at –80°C after initial gross pathological examination. Portions of the primary tumor were cut into 7-μm sections, stained with H&E, and examined by light microscopy to assess neoplastic cellularity. Additional 12-μm sections were cut and placed in a mixture of 1% SDS and proteinase K at 48°C overnight. Tumors with a low

Received 3/23/01; accepted 10/4/01.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

\textsuperscript{1} Supported by Lung Spore Grant (CA-58184-01) and Public Health Service Grant K08 CA76452-01 (National Cancer Institute).

\textsuperscript{2} To whom requests for reprints should be addressed, at Department of Surgery, Box SURG, University of Rochester, 601 Elmwood Avenue, Rochester, NY 14642.

\textsuperscript{3} MGMT, O\textsubscript{6}-methylguanine DNA methyltransferase; MSPCR, methylation specific PCR; NSCLC, non-small cell lung cancer; BPDE, benzo(a)pyrene diol epoxide; PAH, polycyclic aromatic hydrocarbons; NNK, 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone.

Received on December 25, 2017. © 2001 American Association for Cancer Research.

Downloaded from cancerres.aacrjournals.org on December 25, 2017. © 2001 American Association for Cancer Research.
neoplastic cellularity (<70%) were additionally microdissected to remove contaminating normal cells. DNA was extracted with phenol/chloroform and precipitated with ethanol.

**p53 and K-ras Gene Sequencing.** Mutation analysis of the p53 gene was performed on all 92 lung cancers by both direct dideoxy nucleotide sequencing and the GeneChip p53 assay (Affymetrix, Inc., Santa Clara, CA) as reported previously (28). A 1.8-kb fragment of the p53 gene (exons 5–9) was amplified from primary tumor DNA in all 92 patients by PCR (21). The PCR products were purified and sequenced directly using cycle sequencing (Amplicycle sequencing kit; Perkin-Elmer, Branchburg, NJ), and the products of the sequencing reactions were then separated by electrophoresis and exposed to film. Exons 2–11 of the p53 gene were also sequenced using the GeneChip p53 assay as described (28). Mutation analysis of the K-ras gene was performed in patients with primary adenocarcinoma of the lung using a mutation ligation assay as described previously (30).

**MSPCR.** The methylation status of the CpG island of the MGMT gene was determined for each tumor sample using MSPCR (1, 18). Up to 1 μg of DNA was denatured by NaOH and modified using sodium bisulfite. Bisulfite chemically modifies unmethylated, but not methylated, cytosines to uracil. After modification, DNA was purified using Wizard DNA purification resin (Promega), treated with NaOH, and precipitated with ethanol. DNA was resuspended in water and amplified by MSPCR using primers specific for either modified or unmodified DNA. Primer sequences for the methylated reaction were 5′-TTT CGA CGT TCG TAG GTT TCC GC-3′ (sense) and 5′-CGA CTC TTC CGA AAA CGA AAC G-3′ (antisense) and for the unmethylated, modified reaction were 5′-TTT GTG TTT TTA GTG TTT TAG GTT GTT GT-3′ (sense) and 5′-AAC TCC ACA CTC TTC CAA AAA CAA AAC A-3′ (antisense). The annealing temperature was 61°C. The cell line SW480, known to be methylated at the MGMT gene locus, was used as a positive control. Human placental DNA and water were used as negative controls. After MSPCR, 20 μl of each PCR reaction were loaded onto non-denaturing 6% polyacrylamide gels, stained with ethidium bromide, and visualized under UV light.

**MGMT Immunohistochemistry.** Fresh frozen specimens were embedded in Optimum Cold Temperature Medium (Tissue-Tek, Miles, Elkhart, IN), sectioned, and mounted on lysine-coated slides. Immunohistochemistry was performed using the streptavidin biotinylated peroxidase complex method using a mouse MGMT monoclonal antibody (clone mT3.1; NeoMarkers, Inc., Fremont, CA) in a 1:100 dilution. This antibody has been shown to correlate with MGMT promoter hypermethylation and to preferential carcinogen binding of MGMT promoter hypermethylation, 80% of the 10 methylated tumors had detectable MGMT protein by immunohistochemistry. Only 2 of the 10 methylated tumors had detectable MGMT protein by immunohistochemistry.

**MGMT Inactivation and p53 Mutations.** p53 mutations were present in 55 of the 92 tumors (60%) selected for analysis (Fig. 2). MGMT promoter inactivation was present significantly more often in tumors with a G to A transition in p53 (64%) than in tumors with other p53 mutations (27%, P = 0.02) or in tumors with wild-type p53 (18%, P = 0.006; Fig. 3). G to A mutations in p53 were present in only 5 of the 65 (8%) unmethylated tumors (Fig. 4). In contrast, G to A mutations were found in 9 of the 27 (33%) tumors with MGMT promoter methylation and in 45% of the methylated tumors with a p53 mutation. No difference in the frequency of non-G to A p53 mutations was observed among unmethylated and methylated tumors (Table 1). Thus, MGMT inactivation is linked only to G to A mutations in p53 and not to other p53 mutations.

One-third of p53 mutations in NSCLC occurs at CpG sites (21, 23). Mutations at CpG sites have been attributed to spontaneous deamination of methylated cytosines and to preferential carcinogen binding at these sites (22, 25). Mutations (12 of 55) in this set of tumors were at CpG sites. In tumors with MGMT promoter hypermethylation, 80% (four of five) of the mutations at CpG sites were G to A transitions (Table 1). In contrast, 86% (six of seven) of the p53 mutations at CpG sites in tumors with unmethylated MGMT were either G to T or G to C transversions (Fig. 4).

K-ras mutations were present in 17 of the 44 lung adenocarcinomas. No relationship between MGMT promoter hypermethylation and....

---

**Fig. 1.** MSPCR of MGMT. Ladder (100 bp) is shown at left as a molecular weight marker. Product in Lane M indicates the presence of hypermethylation in the MGMT promoter, whereas product in Lane U indicates the absence of promoter hypermethylation. MGMT promoter hypermethylation was present in tumors 1564 and 961 and absent in 1199, 934, 1504, and 829. NL, unmethylated human placental DNA; SW480, methylated positive control cell line; H2O, water.

**Fig. 2.** Dideoxynucleotide sequencing of exon 7 of the p53 gene. A, point mutation (arrow) at codon 241 in sample 1750 (TCC to TTC, G to A on nontranscribed strand). B, point mutation (arrow) at codon 248 in methylated tumor, M32 (CGG to TGG, G to A at CpG site on nontranscribed strand).
the prevalence of K-ras mutations was observed. Only 1 of the 17 K-ras mutations was a G to A mutation.

Discussion

Gene inactivation through promoter hypermethylation has been linked both directly and indirectly to several critical events in neoplasia (10). MGMT hypermethylation was present in ~29% of NSCLC in this study and was significantly more common in lung adenocarcinoma and poorly differentiated tumors. In addition, MGMT expression through promoter methylation was strongly associated with G to A mutations in the p53 gene. The difference in p53 mutation spectra among unmethylated and methylated tumors suggests that MGMT protects against G to A mutations in p53 when present, but loss of MGMT through hypermethylation contributes to a large fraction of p53 mutations.

Low or absent MGMT expression has been identified in both normal and neoplastic tissue (5, 6, 8). Methylation of the MGMT promoter is present in nearly all cancers with loss of expression (1). MGMT methylation is common in NSCLC (24%), colon cancer (38%), and gliomas (38%); occurs to a lesser degree in lymphomas (25%) and pancreatic cancer (11%); and is absent in breast, ovarian, and endometrial cancer (1). MGMT plays a major role in repairing DNA damage from alkylating chemotherapeutic agents and may be responsible for determining the clinical response of certain tumors to these drugs (32, 33). MGMT methylation was also associated with improved survival in patients with gliomas after treatment with the alkylating agent carmustine (33).

MGMT inactivation may be an early event in tumorigenesis conferring an increased susceptibility to nitrosamines and alkylating agents. The MGMT gene is methylated in small (<1 cm) colorectal adenomas preceding the appearance of K-ras mutations, which are only present in larger adenomas or invasive tumors (18). We have not examined preneoplastic lung lesions for the presence of MGMT methylation. However, the prevalence of both MGMT methylation and p53 mutations is similar in stage I and III tumors, suggesting that both these events precede the development of an invasive cancer. Furthermore, the increase in frequency of G to A mutations from unmethylated (9%) to methylated (45%) tumors suggests that MGMT inactivation may play a role in a significant fraction of these mutations in lung cancer.

Cigarette smoking significantly increases the frequency of p53 mutations in NSCLC (21). Tobacco smoke is a complex mixture of carcinogens, including PAHs, nitrosamines, and other alkylating agents (34). BPDE and a series of other activated PAHs preferentially form DNA adducts at CpG site guanines, which are also the sites of highest mutation frequency in lung cancer (26). A variety of carcinogenic adducts have also been shown to have similar DNA binding preferences (25). These data suggest that other DNA-reactive compounds and DNA adducts derived from tobacco smoke may have similar effects (34). Along with the PAHs, the data supporting a major role for individual carcinogens in lung cancer is strongest for the tobacco-specific nitrosamine NNK (34). NNK is a potent lung carcinogen in rodents and forms methyl adducts at the O6 position of guanine and pyridyloxobutyl adducts, both repaired by MGMT (34, 35). Expression of human MGMT reduces the frequency of lung tumors in an NNK-induced mouse model of lung cancer (36). Cigarette smoking is known to increase MGMT expression in both normal and neoplastic lung tissue, suggesting that MGMT may protect the lung from carcinogen-induced guanine alkylation (4, 7).
Both strands of the human p53 gene are extensively methylated at every CpG site (37, 38). The majority of p53 mutations at CpG sites in human cancer is G to A transitions (23). These mutations have been attributed to endogenous deamination of methylated cytosine to thymine, although definitive evidence of this occurring in vivo or in the lung is lacking (22, 23, 34, 37, 38). Chen et al. (25) demonstrated that cytosine methylation enhances guanine alkylation by a variety of bulky carcinogens (BPDE, aflatoxin B1 8,9-epoxide, benzo[g]chrysene diol epoxide, and N-acetoxy-2-acetylaminofluorene). Slow repair of bulky DNA adducts along the nontranscribed strand also plays a role in the high frequency of p53 mutations at CpG sites (27). Cytosine methylation, which is present at all CpG sites in the p53 gene, is known to diminish the activity of MGMT (39). Chen et al. (25) have proposed that enhanced carcinogen binding and decreased DNA repair efficiency and not spontaneous deamination of methylated cytosine are the major determinants of p53 mutations at CpG sites. This study supports their observations and additionally suggests that MGMT plays an important role in DNA repair in NSCLC both at CpG and non-CpG sites and that MGMT inactivation leads to an increase in G to A p53 mutations in this disease.

Inactivation of the MGMT gene through promoter hypermethylation is a common event in NSCLC. The shift in the p53 mutational spectrum observed in methylated tumors suggests that MGMT promoter hypermethylation precedes mutation of the p53 gene in lung tumorigenesis. Because G to A mutations account for >40% of the p53 mutations in human cancer (23), this study supports the notion that MGMT inactivation may play a role in many of these mutations. An improved understanding of the factors contributing to MGMT promoter hypermethylation may yield novel therapeutic targets for chemoprevention. In addition, determining the methylation status of MGMT may help select patients likely to respond to commonly used alkylating agents (e.g., cisplatin and carboplatin) in NSCLC as shown recently for gliomas (33).


O⁶-Methylguanine-DNA Methyltransferase Promoter Hypermethylation Shifts the p53 Mutational Spectrum in Non-Small Cell Lung Cancer

Patrick Wolf, Ying Chuan Hu, Kara Doffek, et al.


Updated version
Access the most recent version of this article at:
http://cancerres.aacrjournals.org/content/61/22/8113

Cited articles
This article cites 38 articles, 19 of which you can access for free at:
http://cancerres.aacrjournals.org/content/61/22/8113.full#ref-list-1

Citing articles
This article has been cited by 11 HighWire-hosted articles. Access the articles at:
http://cancerres.aacrjournals.org/content/61/22/8113.full#related-urls

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, use this link
http://cancerres.aacrjournals.org/content/61/22/8113. Click on "Request Permissions" which will take you to the Copyright Clearance Center’s (CCC) Rightslink site.