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Increased Expression of Deoxyribonucleic Acid Methyltransferase Gene in Human Astrocytic Tumors

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Abstract

The relationship between the grade of astrocytic tumor and the expression of deoxyribonucleic acid methyltransferase (DNA-MTase) gene was examined. The levels of DNA-MTase messenger ribonucleic acid (mRNA) were measured by semiquantitative reverse transcriptase-polymerase chain reaction in surgical specimens from 12 astrocytic tumors (4 astrocytomas, 6 anaplastic astrocytomas, and 2 glioblastomas) and two normal brain tissues, and in four glioma cell lines. Compared to normal brain tissues, the levels of DNA-MTase mRNA were increased by 16- to 55-fold in low grade astrocytomas, and significantly increased by 200- to 4500-fold in high grade astrocytomas (anaplastic astrocytomas and glioblastomas) and more than 4500-fold in glioma cell lines. In situ hybridization with paraffin-embedded surgical specimens of human astrocytic tumors showed DNA-MTase mRNA was abundantly expressed in high grade astrocytomas. The detection of increased DNA-MTase expression in astrocytic tumor indicates involvement in the tumorigenesis and suggests that blocking of this change with specific inhibitors may offer new therapeutic strategies for malignant astrocytic tumors.

Key words: glioma, deoxyribonucleic acid methyltransferase, semiquantitative reverse transcriptase-polymerase chain reaction, in situ hybridization

Introduction

Tumor progression involves interactions between multiple adverse genetic events, including chromosomal translocations, deletions, amplifications, and point mutations.[22,39] The etiology of these progression changes may involve the abnormal pattern of deoxyribonucleic acid (DNA) methylation that occurs consistently in human tumors and is manifested as widespread genomic hypomethylation and regional areas of abnormally increased methylation.[17-19] Abnormal methylation has been associated with changes of chromatin structure[1,12] that can inhibit gene expression.[8] DNA methylation could also result in changes in DNA sequences, since methylated cytosine is a highly mutable base in the eukaryotic genome.[10,18,31]

Abnormal patterns of DNA methylation in neoplastic cells could be evaluated by measuring the increase in DNA methyltransferase (DNA-MTase) activity associated with tumor evolution.[13] Expression of the DNA-MTase gene, which encodes the enzyme that catalyzes cytosine methylation at CpG sites, is abnormally high in tumor cells and increases throughout the progression stages of human colon cancer.[13] Increased expression of the DNA-MTase gene occurs in human glioma cells and may be involved in chromosomal instability and malignant transformation in human glioma. Increases in DNA-MTase gene expression may alter DNA methylation patterns and play a role in the genetic instability of human astrocytic tumors. However, no correlation between abnormal changes in DNA-MTase gene expression and the grade of astrocytic tumor has been established.

This study examined the relationship between the grade of astrocytic tumor and the expression of DNA-MTase gene.

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Materials and Methods

I. Cell culture and tissue collection

Human high grade malignant glioma cell lines, U373MG, T98G, U251MG, and U87MG, were obtained from the Japan Cancer Research Resource Bank Inc. (Tokyo), Institute for Fermentation (Osaka), and Riken Cell Bank Inc. (Tsukuba, Ibaraki). Cells were grown to confluence in Dulbecco's modified Eagle's medium supplemented with 2 mM L-glutamine and 10% fetal bovine serum.

Tumor samples and normal human brain tissues were obtained from 14 patients undergoing surgery at Kanazawa University Hospital and affiliated hospitals. The patients gave permission for tissue collection. All tumors were classified on the basis of the World Health Organization (WHO) criteria for tumors of the central nervous system.[21] There were four cases of low grade astrocytoma (WHO grade II), six cases of anaplastic astrocytoma (WHO grade III), and two cases of primary type glioblastoma multiforme (WHO grade IV). Normal human brain tissues were obtained from two patients at lobectomy and autopsy. Samples were immediately frozen in liquid nitrogen and kept at −80°C. Paraformaldehyde fixed (4%), paraffin-embedded samples were used for in situ hybridization.

II. Reverse transcriptase-polymerase chain reaction (RT-PCR) and Southern blot analysis

Total ribonucleic acid (RNA) was extracted by acid guanidinium thiocyanate-phenol-chloroform extraction (RNAzol B kit; Biotex Laboratories Inc., Edmonton, Canada).[9] Expression of the transcript for DNA-MTase was measured by RT-PCR (GeneAmp Thermostable rTth reverse transcriptase RNA PCR kit; Perkin Elmer Cetus, Norwalk, Conn., U.S.A.). The PCR product yield does not quantitatively reflect the amount of the initial template DNA after the reaction reaches the plateau. Therefore, the product was analyzed by measuring the amount of radioactivity in the product after every five cycles of reaction to avoid the plateau effect. The internal control measured human glyceraldehyde 3-phosphate dehydrogenase (G3PDH) messenger RNA (mRNA) under the same conditions using the same method. Sense and antisense primers were complementary to reported complementary DNA sequences for DNA-MTase[42]: 5'-TAGAGTGGGAATGGCAGATG-3' [351-370] and 5'-GCGGTCTAGCAACTCGTTCT-3' [627-646], respectively. Sense and antisense primers for G3PDH were 5'-ACCACAGTCCATGCCATCAC-3' [586-605] and 5'-TCCACCACCCTGTTGGCTGTA-3' [1018-1037], respectively (Clontech Laboratories Inc., Palo Alto, Cal., U.S.A.). The RT reaction was performed at 70°C for 10 minutes and amplification was allowed for indicated cycles of 94°C for 1 minute, 60°C for 2 minutes for DNA-MTase, and 94°C for 45 seconds, 60°C for 45 seconds, 72°C for 1 minute for G3PDH. Samples were collected after every five cycles of reaction and electrophoresed in 2.5% agarose gels and transferred to Nytran-modified nylon filters by capillary action.

Southern blotting was performed as described.[32] Southern blotting used [α^{32}P]deoxycytidine triphosphate-labeled oligonucleotide probes, 5'-TAGCCCCAGGATTACAAGGAAAAGCACCAG-3' [456-485] for DNA-MTase and 5'-TCTCCTCTGACTTCAACAGCGACACCCACT-3' [3388-3417] for G3PDH. The intensity of radioactivity of each DNA band was measured using a laser densitometric bioimage analyzer (BAS2000; FUJIX, Tokyo).

III. In situ hybridization

The DNA-MTase RT-PCR product obtained from U87MG was cloned to pGEM-T vector (Promega, Madison, Wis., U.S.A.) and sequenced using an ALF DNA sequencer (Pharmacia, Piscataway, N.J., U.S.A.). The DNA-MTase RNA probes were synthesized and labeled by digoxigenin-labeled deoxyuridine triphosphate using a RNA color kit (Amersham Life Science Inc., Arlington Heights, Ill., U.S.A.) according to the manufacturer's instructions. Sections (4 μm thick) of 4% paraformaldehyde-fixed, paraffin-embedded tissues were mounted on poly-L-lysine-coated slides. The slides were dewaxed and the sections were pretreated with 0.1N HCl (pH 8.0) and 10 μg/ml proteinase K (Takara, Shiga) at 37°C for 20 minutes before hybridization, acetylated (0.25% acetic anhydride in 0.1 M triethanolamine, pH 8.0) for 10 minutes, and incubated with hybridization buffer containing 500 ng/μl of labeled RNA probe in a moist chamber at 60°C overnight. The slides were washed and incubated in 1% blocking reagent for 60 minutes, placed in a moist chamber and incubated with a 1:1000 dilution of anti-alkaline phosphate conjugate for 60 minutes, rinsed with Tris-buffered saline, and incubated with nitroblue tetrazolium and 5-bromo-4-chrolo-3-indoxyl phosphate in a moist chamber according to the manufacturer's instructions for the RNA color kit. Additionally, the slides were counterstained with methylgreen, air dried, and mounted with glycerol/phosphate buffered saline.

Results

Table 1 shows DNA-MTase expression in the various glioma cell lines and surgical specimens. Deter-

Table 1 Sample list and relative amount of deoxyribonucleic acid methyltransferase (DNA-MTase) messenger ribonucleic acid (mRNA)

Sample	Relative amount of DNA-MTase mRNA*
Glioma**	
Astrocytoma	
Case 1	0.055
Case 2	0.030
Case 3	0.016
Case 4	0.035
Anaplastic astrocytoma	
Case 5	0.325
Case 6	1.042
Case 7	0.217
Case 8	0.687
Case 9	1.324
Case 10	4.540
Glioblastoma	
Case 11	0.823
Case 12	2.009
Glioma cell lines	
U373MG	4.493
T98G	4.955
U251MG	7.652
U87MG	30.868
Normal human brain	
Sample A	0.001
Sample B	0.001

*Analyzed by the quantitative reverse transcriptase-polymerase chain reaction method and standardized using glyceraldehyde 3-phoshate dehydrogenase mRNA as an internal control. **According to World Health Organization classification.

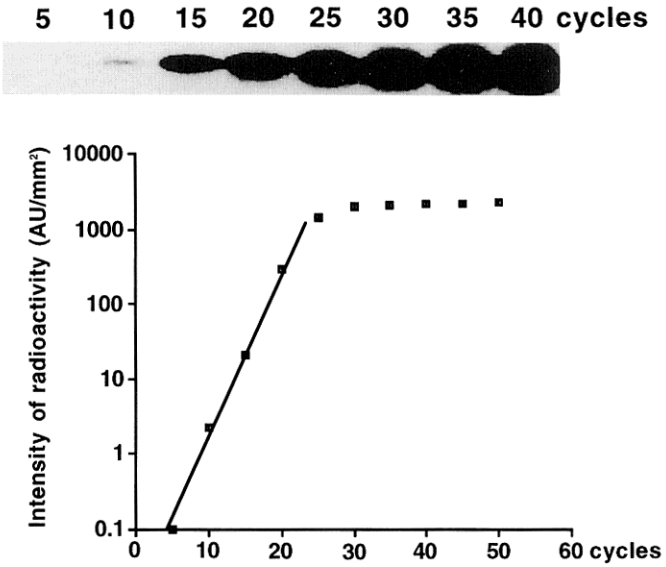

Fig. 1 Semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of deoxyribonucleic acid methyltransferase messenger ribonucleic acid (RNA) for the determination of the exponential range of cycle numbers. All sequential cycles were initiated with 200 ng of total RNA from U251MG glioma cells. *upper*: Autoradiograms of Southern blots performed on RT-PCR products after 5–40 cycles. *lower*: Signal intensities of RT-PCR products expressed as the arbitrary logarithm values of intensity of radioactivity and plotted against the numbers of amplification cycles. Radioactivities of hybridization bands were measured with a Fuji BAS2000 BioImage analyzer and described in arbitrary units (AU) per mm².

mination of the exponential range of cycle numbers for semiquantitative RT-PCR was performed with 200 ng of total RNA from U251MG glioma cells (Fig. 1) and surgical specimens (Fig. 2). For comparison of tumor grade and DNA-MTase mRNA expression, each sample was transcribed and amplified by 20 cycles in PCR from 200 ng of total RNA of surgical specimens and cultured glioma cells (Fig. 3). The relative amount of DNA-MTase mRNA was standardized by the amount of G3PDH mRNA. The 296 base pair fragments from DNA-MTase mRNA were recognized in all normal brains and glioma specimens. Compared to normal brain tissues, the levels of DNA-MTase mRNA were increased by 16- to 55-fold in low grade astrocytomas, and increased significantly ($p < 0.05$ by *t*-test) by 200- to 4500-fold in high grade astrocytomas (anaplastic astrocytomas and glioblastomas) and more than 4500-fold in glioma cell lines. In situ hybridization with paraffin-embedded surgical specimens of human astrocytic tumors showed DNA-MTase mRNA was abundantly expressed in high grade astrocytomas (Fig. 4).

Discussion

An important aspect of the abnormal pattern of DNA methylation seen in tumor progression is the abnormal de novo methylation[1,5] of normally unmethylated clusters of cytosine-guanosine dinucleotides, the C + G rich areas termed "CpG islands" which are usually located in the 5' region of genes.[7] Local increases in DNA methylation in cancer cells can be strategically placed in the CpG islands, including regions on the short arm of chromosome 11[12] where tumor suppressor genes are known to reside,[33,40] on chromosome 16 in HeLa cells[2] and on multiple areas in tumor cells.[4,14,23,25–27,30,37] DNA-MTase may also act directly as an endogenous mutagen by causing enzymatic deamination of cytosine at a very high frequency.[34] The increased

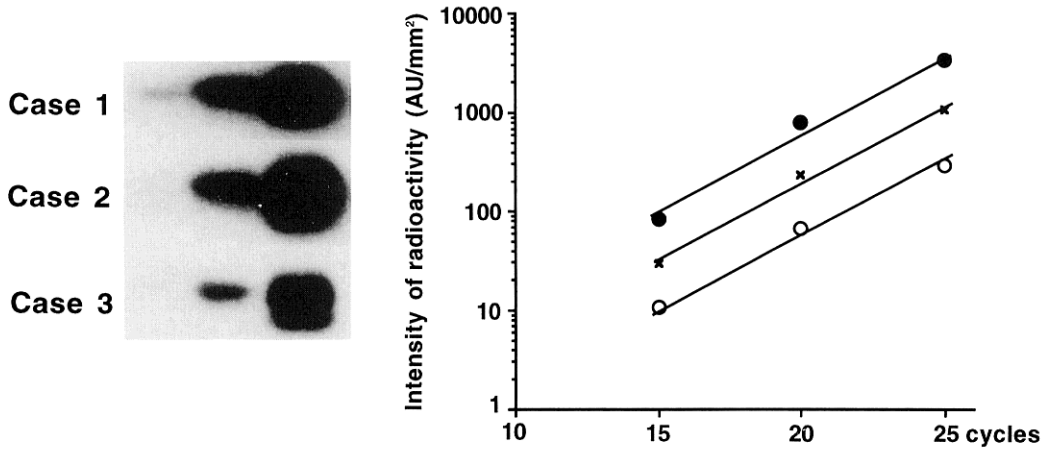

Fig. 2 Semiquantitative reverse transcriptase-polymerase chain reaction (RT-PCR) analysis of deoxyribonucleic acid methyltransferase messenger ribonucleic acid in surgical specimens from three patients. *left*: Autoradiograms of Southern blots performed on RT-PCR products after 15–25 cycles. *right*: Signal intensities of RT-PCR products expressed as the arbitrary logarithm values of intensity of radioactivity and plotted against the numbers of amplification cycles. Radioactivities of hybridization bands were measured with a Fuji BAS2000 BioImage analyzer and described in arbitrary units (AU) per mm^2: ○, Case 1; ×, Case 2; ●, Case 3.

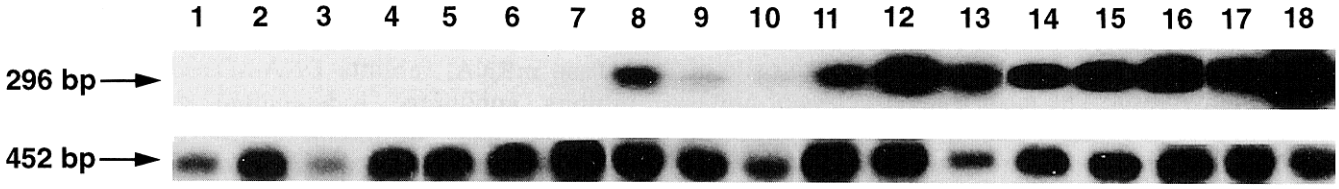

Fig. 3 Autoradiograms of Southern blots performed on reverse transcriptase-polymerase chain reaction (PCR) products from 200 ng of total ribonucleic acid (RNA) from surgical specimens and cultured glioma cells. Each sample was transcribed and amplified by 20 cycles of PCR. Upper lanes show 296 base pair (bp) fragments from deoxyribonucleic acid methyltransferase messenger RNA (mRNA) and lower lanes show 452 bp fragments from glyceraldehyde 3-phosphate dehydrogenase mRNA as an internal control: lanes 1 and 2, normal brains; lanes 3–6, astrocytomas; lanes 7–12, anaplastic astrocytomas; lanes 13 and 14, glioblastomas; lanes 15–18, glioma cell lines.

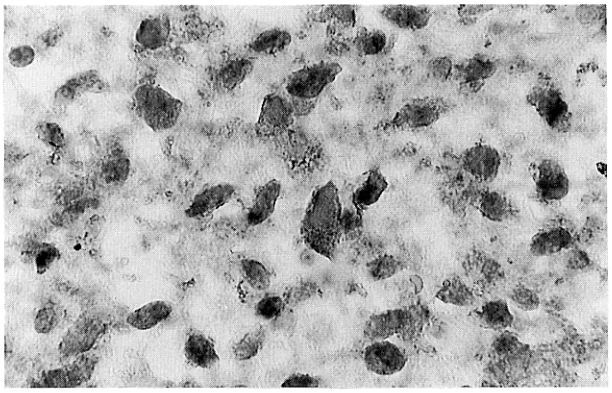

Fig. 4 Photomicrograph showing in situ hybridization with antisense ribonucleic acid (RNA) probe for deoxyribonucleic acid methyltransferase messenger RNA in a glioblastoma specimen. Original magnification, ×400.

DNA-MTase activity is critical for maintaining the neoplastic character in adenocortical carcinoma and is reversible by a phosphorothioate-modified, antisense oligodeoxynucleotide.[29] The present study shows the correlation between increased DNA-MTase activity and the grade of astrocytic tumor, suggesting that abnormal methylation may be associated with the progression stages of glioma. Increases in DNA-MTase gene expression are known to precede development of colonic neoplasia and continue during the progression of colonic neoplasms.[13] Increased DNA-MTase gene expression also occurs in hypertrophic alveolar type II cells of carcinogen-treated A/J mice,[6] indicating that elevated levels of expression may be a biomarker for premalignancy.

DNA-MTase can convert 5-methylcytosine in DNA to thymine.[3,41] The target cytosine is thought to be flipped out of the double helix, and the C6 of the base is attacked by cysteine thiolate in the enzyme. Subsequent charge delocalization is expected to create two possible enamine-containing intermediates. Protonation of these intermediates should result in the formation of a positively charged iminium species. The iminium ion should readily undergo hydrolytic deamination to form thymine. This reaction creates a mutational hot spot at the site of DNA methylation. The most frequent class of mutations is C to T changes within CG dinucleotides of the tumor suppressor gene p53.[41] This reaction is expected to contribute to mutations at CG dinucleotides in precancerous cells. Mutated p53 gene in malignant glioma is often associated with progression and recurrence of malignancy, and these events are closely linked with increased resistance to both chemotherapy and radiation.[15,16] In addition, transcriptional repression of p16/CDKN2 in gliomas is mediated by aberrant methylation of the CpG islands.[11] These islands are important for maintenance of open transcriptional confirmation around genes, are normally unmethylated in adult cells except on the increased X chromosome,[1,19] and participate in a closed chromatin pattern when abnormally methylated in cancer[12] or immortalized cells.[1]

The correlation between increased DNA-MTase activity and the grade of astrocytic tumor suggests that this enzyme has a fundamental role in the initiation and progression of glioma. Three possible mechanisms have been suggested for elevated levels of DNA-MTase in tumors. First, elevated levels of DNA-MTase might result in disruption of the appropriate gene expression profile of a cell to inactivation of tumor suppressor genes.[38] Second, high levels of DNA-MTase might have a direct effect on the origins of replication.[35,36] Third, methylated cytosines are hot spots for mutation.[10,18,31] Abnormal methylation might account for transcriptional inactivation of the suppressor genes such as p53 gene and subsequent malignant transformation in glioma. This mechanism for gene inactivation during glioma development might have important clinical implications.

More data are required to determine which mechanism is critical in the genesis and maintenance of malignant glioma, but DNA-MTase may be a candidate target for an anti-glioma study. The deoxycytidine analog 5-aza-2'-deoxycytidine (5-azadCyd) has been widely used as a DNA methylation inhibitor to experimentally induce gene expression and cellular differentiation.[28] Cellular DNA-MTase, rather than the secondary demethylation of genomic DNA, is the primary mediator of 5-azadCyd cytotoxicity.[20] The incorporation of the 5-azadCyd into DNA blocks DNA methylation and can result in the activation of specific genes, such as tumor suppressor genes. A pilot phase I–II study in patients with metastatic lung cancer has suggested that 5-azadCyd has clinical activity.[24] Furthermore, antisense oligodeoxynucleotide directed against DNA-MTase mRNA reduces the level of DNA-MTase mRNA, inhibits DNA-MTase activity, and inhibits anchorage independent growth of Y1 adrenocortical carcinoma cells.[29] Expression of antisense to DNA-MTase mRNA induces DNA demethylation and inhibits tumorigenesis. The detection of increased DNA-MTase expression in astrocytic tumors indicates involvement in tumorigenesis and suggest that blocking of the deleterious effects of this change with specific inhibitors may offer new therapeutic strategies for malignant astrocytic tumors.

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References

1) Antequera F, Boyes J, Bird A: High levels of de novo methylation and altered chromatin structure at CpG islands in cell lines. *Cell* 62: 503-514, 1990
2) Antequera F, Macleod D, Bird AP: Specific protection of methylated CpGs in mammalian nuclei. *Cell* 58: 509-517, 1989
3) Bandaru B, Gopal J, Bhagwat AS: Overproduction of DNA cytosine methyltransferases causes methylation and C→T mutations at non-canonical sites. *J Biol Chem* 271: 7851-7859, 1996
4) Baylin SB, Hoppener JWM, Bustros A, Steenbergh PH, Lips CJM, Nelkin BD: DNA methylation pattern of the calcitonin gene in human lung cancers and lymphomas. *Cancer Res* 46: 2917-2922, 1986
5) Baylin SB, Makos M, Wu JJ, Yen RWC, de Bustros A, Vertino P, Nelkin BD: Abnormal patterns of DNA methylation in human neoplasia: potential consequences for tumor progression, *in*: *Cancer Cells*. Cold Spring Harbor, NY, Cold Spring Harbor Laboratory Press, 1991, pp 383-390
6) Belinsky SA, Nikula KJ, Baylin SB, Issa JP: Increased cytosine DNA-methyltransferase activity is target-cell-specific and an early event in lung cancer. *Proc Natl Acad Sci U S A* 93: 4045-4050, 1996
7) Bird AP: CpG-rich island and the function of DNA methylation. *Nature* 321: 209-213, 1986
8) Boyes J, Bird A: DNA methylation inhibits transcription indirectly via a methyl-CpG binding protein. *Cell* 64: 1123-1134, 1991
9) Chomczynski P, Sacchi N: Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. *Anal Biochem* 162: 156-159, 1987
10) Cooper DN, Youssoufian H: The CpG dinucleotide and human genetic disease. *Hum Genet* 78: 151-155, 1988
11) Costello JF, Berger MS, Huang HS, Cavenee WK: Silencing of p16/CDKN2 expression in human gliomas by methylation and chromatin condensation. *Cancer Res* 56: 2405-2410, 1996
12) de Bustros A, Nelkin BD, Silverman A, Ehrlich G, Poiesz B, Baylin SB: The short arm of chromosome 11 is a "hot spot" for hypermethylation in human neoplasia. *Proc Natl Acad Sci U S A* 85: 5693-5697, 1988
13) el-Deiry WS, Nelkin BD, Celano P, Yen RC, Falco JP, Hamilton SR, Baylin SB: High expression of the DNA methyltransferase gene characterizes human neoplastic cells and progression stages of colon cancer. *Proc Natl Acad Sci U S A* 88: 3470-3474, 1991
14) Falette NS, Fuqua SAW, Chamness GC, Chesh MS, Greene GL, McGuire WL: Estrogen receptor gene methylation in human breast tumors. *Cancer Res* 50: 3974-3978, 1990
15) Hayashi Y, Yamashita J, Yamaguchi K: Timing and role of p53 gene mutation in the recurrence of glioma. *Biochem Biophys Res Commun* 180: 1145-1150, 1991
16) Iwadate Y, Fujimoto S, Tagawa M, Namba H, Sueyoshi K, Hirose M, Sakiyama S: Association of p53 gene mutation with decreased chemosensitivity in human malignant gliomas. *Int J Cancer* 69: 236-240, 1996
17) Jones PA: DNA methylation and cancer. *Cancer Res* 46: 461-466, 1986
18) Jones PA: DNA methylation errors and cancer. *Cancer Res* 56: 2463-2467, 1996
19) Jones PA, Buckley JD: The role of DNA methylation in cancer. *Adv Cancer Res* 54: 1-23, 1990
20) Juttermann R, Li E, Jaenisch R: Toxicity of 5-aza-2'-deoxycytidine to mammalian cells is mediated primarily by covalent trapping of DNA methyltransferase rather than DNA demethylation. *Proc Natl Acad Sci U S A* 91: 11797-11801, 1994
21) Kleihues P, Burger PC, Scheithauer BW: *Histological Typing of Tumors of the Central Nervous System, ed 2*. Berlin, Springer-Verlag, 1991, 100 pp
22) Lang FF, Miller DC, Koslow M, Newcomb EW: Pathways leading to glioblastoma multiforme: a molecular analysis of genetic alterations in 65 astrocytic tumors. *J Neurosurg* 81: 427-436, 1994
23) Makos M, Nelkin BD, Lerman MI, Latif F, Zbar B, Baylin SB: Distinct hypermethylation patterns occur at altered chromosome loci in human lung and colon cancer. *Proc Natl Acad Sci U S A* 89: 1929-1933, 1992
24) Momparler RL, Bouffard DY, Momparler LF, Dionne J, Belanger K, Ayoub J: Pilot phase I-II study on 5-aza-2'-deoxycytidine (Decitabine) in patients with metastatic lung cancer. *Anticancer Drugs* 8: 358-368, 1997
25) Ohtani-Fujita N, Fujita T, Osifichin NE, Robbins PD, Sakai T: CpG methylation inactivates the promotor activity of the human retinoblastoma tumor-suppressor gene. *Oncogene* 8: 1063-1067, 1993
26) Ottaviano YL, Issa JP, Parl FF, Smith HS, Baylin SB, Davidson NE: Methylation of the estrogen receptor gene CpG island marks loss of estrogen receptor expression in human breast cancer cells. *Cancer Res* 54: 2552-2555, 1994
27) Pieretti M, Powell DE, Gallion HH, Conway PS, Case EA, Turker MS: Hypermethylation at a chromosome 17 "hot spot" is a common event in ovarian cancer. *Hum Pathol* 26: 398-401, 1995
28) Rainier S, Feinberg AP: Capture and characterization of 5-aza-2'-deoxycytidine-treated C3H/10T1/2 cells prior to transformation. *Proc Natl Acad Sci U S A* 85: 6384-6388, 1988
29) Ramchandani S, MacLeod AR, Pinard M, von Hofe E, Szyf M: Inhibition of tumorigenesis by a cytosine-DNA, methyltransferase, antisense oligodeoxynucleotide. *Proc Natl Acad Sci U S A* 94: 684-689, 1997
30) Ribieras S, Song WX, Martin V, Lointier P, Frappart L, Dante R: Human breast and colon cancers exhibit alteration of DNA methylation patterns at several DNA segments on chromosomes 11p and 17p. *J Cell Biochem* 56: 86-96, 1994
31) Rideout WM III, Coetzee GA, Olumi AF, Jones PA:

5-Methylcytosine as an endogenous mutagen in the human LDL receptor and p53 genes. *Science* 249: 1288–1290, 1990
32) Sambrook J, Fritsch EF, Maniatis T: *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor, NY, Cold Spring Harbor Laboratory Press, 1989, 100 pp
33) Scrable H, Cavenee W, Ghavimi F, Lovell M, Morgan K, Sapienza C: A model for embryonal rhabdomyosarcoma tumorigenesis that involves genome imprinting. *Proc Natl Acad Sci U S A* 86: 7480–7484, 1989
34) Shen J, Rideout WM III, Jones PA: High frequency mutagenesis by a DNA methyltransferase. *Cell* 71: 1073–1080, 1992
35) Szyf M: DNA methylation properties: consequences for pharmacology. *Trends Pharmacol Sci* 15: 233–238, 1994
36) Szyf M: The DNA methylation machinery as a target for anticancer therapy. *Pharmacol Ther* 70: 1–37, 1996
37) Tulchinsky E, Grigorian M, Tkatch T, Georgiev G, Lukanidin E: Transcriptional regulation of the mst1 gene in human lymphoma cells: the roll of DNA methylation. *Biochim Biophys Acta* 1261: 243–248, 1995
38) Wales MM, Biel MA, el Deiry W, Nelkin BD, Issa JP, Cavenee WK, Kuerbitz SJ, Baylin SB: p53 activates expression of HIC-1, a new candidate tumour suppressor gene on 17p13. 3. *Nat Med* 1: 570–577, 1995
39) Weinberg RA: Prospects for cancer genetics. *Cancer Surv* 25: 3–12, 1995
40) Weissman BE, Saxon PJ, Pasquale SR, Jones GR, Geiser AG, Stanbridge EJ: Introduction of a normal human chromosome 11 into a Wilms' tumor cell line controls its tumorigenic expression. *Science* 236: 175–180, 1987
41) Yebra MJ, Bhagwat AS: A cytosine methyltransferase converts 5-methylcytosine in DNA to thymine. *Biochemistry* 34: 14752–14757, 1995
42) Yen RW, Vertino PM, Nelkin BD, Yu JJ, el-Deiry W, Cumaraswamy A, Lennon GG, Trask BJ, Celano P, Baylin SB: Isolation and characterization of the cDNA encoding human DNA methyltransferase. *Nucleic Acids Res* 20: 2287–2291, 1992

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Commentary

This is a carefully done and potentially very important study regarding levels of gene activity and expression for an important enzyme in malignant gliomas. The author studied a total of 12 astrocytic tumors of varying degrees of malignancy and carefully analyzed levels of deoxyribonucleic acid (DNA) methyltransferase by analyzing mRNA using PCR. The hypothesis is that the abnormal tumor cells have increased and abnormal methylation of DNA as part of the malignant process and the increase in the abnormality theoretically would up regulate DNA methyltransferase. They found a clear progression in the expression of the gene and its product with increasing malignancy of the gliomas studied. This same kind of progression in gene activity has been seen in colon cancer, and may very well be a hallmark of the neoplastic process. It would be important to confirm these findings in a study of a large number of primary malignant brain tumors and to test the hypothesis that blocking of this gene activity might inhibit the growth of these malignant neoplasms.

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The authors examined the relationship between the grade of astrocytic tumor and the expression of deoxyribonucleic acid methyltransferase (DNA-MTase) gene, and showed that the levels of DNA-MTase mRNA increased by 16- to 55-fold in low grade astrocytomas, and significantly increased by 200- to 4500-fold in high grade astrocytomas (anaplastic astrocytomas and glioblastomas) and more than 4500-fold in glioma cell lines. They also confirmed the abundant expression of DNA-MTase mRNA in situ hybridization in surgical specimens of human high grade astrocytomas. This report is interesting in that this enzyme has a fundamental role in the initiation and progression of glioma. However, as the authors mention, there are many factors in this mechanism, and a question remains unanswered whether tumors with the highly expressed enzyme have different histological features compared to tumors without the enzyme.

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Dr. Okada and colleagues have clearly documented the increased expression of deoxyribonucleic acid methyltransferase (DNA-MTase) gene in human astrocytic tumors. However, a further concern for most readers will be whether the increased expression of DNA-MTase gene has any relationship to the status of the p53 gene that is important not only in tumorigenesis but also in the malignant progression of certain astrocytic tumors. Therefore, further data re-

garding the p53 status of individual glioma cases and glioma cell lines are required to evaluate the possible contribution of increased activity of DNA-MTase to p53 mutation in human astrocytic tumors. In addition, the authors are asked to elucidate any suspected correlation between increased DNA-MTase activity and inactivation through DNA hypermethylation of some important genes such as p16 and p14 that are often essential for human glial tumorigenesis.

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