Intercellular heterogeneity of expression of the *MGMT* DNA repair gene in pediatric medulloblastoma[1]

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DNA methylation and epigenetic inactivation of the O^6-methylguanine methyltransferase (*MGMT*) gene induces MGMT deficiency, reducing the tumor cell's DNA repair capacity and increasing its susceptibility to alkylating chemotherapeutic agents. Consequently, adult patients whose tumors are deficient in MGMT have better outcomes with alkylator chemotherapy, and *MGMT* methylation has been proposed as a screening marker of deficient tumors. In order to test the feasibility of this approach for medulloblastoma, a common brain tumor in children, we determined the methylation status, mRNA expression pattern, and protein expression of MGMT in a panel of clinical specimens. Methylation-specific polymerase chain reaction analysis revealed methylation of *MGMT* in 28 of 37 tumor samples. Quantitative real-time reverse transcriptase–polymerase chain reaction showed a range of expression of *MGMT* mRNA varying more than 20-fold. However, there was no correlation found between *MGMT* methylation and mRNA expression. Immunohistochemistry demonstrated that all tumors were immunoreactive for MGMT in the nucleus of the medulloblastoma cells in a heterogeneous pattern. The intercell variability of MGMT complement explained the discordance between methylation and expression. Therefore, *MGMT* methylation as determined by methylation-specific polymerase chain reaction cannot be used as a marker for MGMT deficiency in medulloblastoma. Further, these findings support the use of pharmacological MGMT depletion as a rational approach for intensification of alkylator chemotherapy in the treatment of medulloblastoma. *Neuro-Oncology 6, 200–207, 2004 (Posted to Neuro-Oncology [serial online], Doc. 03-056, May 13, 2004. URL http://neuro-oncology.mc.duke.edu; DOI: 10.1215/S1152851703000565)*

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[3] Abbreviations used are as follows: CCNU, lomustine, an abbreviated form of *N*-(2-chloroethyl)-*N*'-cyclohexyl-*N*-nitrosourea; cDNA, complementary DNA; csXRT, craniospinal irradiation; MGMT, O^6-methylguanine methyltransferase; MS-PCR, methylation-specific polymerase chain reaction; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; qRT-PCR, quantitative real-time reverse transcriptase–polymerase chain reaction.

Medulloblastoma is the most common malignant brain tumor in children. Currently, the most effective known chemotherapeutic treatment regimen for standard risk disease includes lomustine (CCNU),[3] a methylating alkylator (Packer et al., 1994). The O^6-methylguanine methyltransferase (MGMT) gene product repairs the DNA damage caused by CCNU by transferring the adducted methyl group to an internal cysteine residue, a process that inactivates the MGMT molecule. Thus, the repair capacity of a cell is stoichiometrically related to its content of MGMT molecules,

and a cell's complement of MGMT is primarily determined by the transcriptional activity of the *MGMT* gene (Fornace et al., 1990).

The transcriptional inactivation of the *MGMT* gene is primarily effected epigenetically in a process marked by DNA methylation. Many types of tumors are deficient in the MGMT protein, including as many as 30% of adult gliomas (Silber et al., 1993, 1998). A similar percentage of medulloblastoma cell lines and a single primary tumor sample have also been found to be MGMT deficient (Esteller et al., 1999; He et al., 1992). However, an earlier series of 14 medulloblastoma/PNET tumors showed a high level of MGMT activity by biochemical assay, which correlated with MGMT protein quantitation by Western blot immunoassay (Hongeng et al., 1997). The amount of activity that was present predicted resistance to CCNU, a component of commonly used therapy. MGMT activity was found to vary over a 100-fold range in another panel of 27 medulloblastomas, although the reasons for this heterogeneity were not explored, and the MGMT protein complement was not quantified (Bobola et al., 2001).

A correlation between decreased levels of tumor MGMT and increased survival of adults with high-grade glioma treated with the alkylator carmustine (BCNU), an agent with a mechanism of action identical to that of CCNU, has been established (Belanich et al., 1996; Jaeckle et al., 1998). In addition, a survival advantage has also been shown for *MGMT* methylation in malignant glioma patients treated with a regimen including carmustine (Esteller et al., 2000a). We therefore characterized *MGMT* methylation, mRNA expression, and protein expression in pediatric medulloblastoma to explore the feasibility of using DNA methylation detection to screen for MGMT deficiency. We also sought to address the rationale for the use of MGMT-depleting agents in the optimization of therapy for medulloblastoma.

Materials and Methods

Methylation-Specific Polymerase Chain Reaction

MGMT methylation status was analyzed by methylation-specific polymerase chain reaction (MS-PCR) (Herman et al., 1996). DNA extracted from pediatric medulloblastoma tumor specimens was denatured with sodium hydroxide and treated with sodium bisulfite according to a method adapted from Frommer et al. (1992), resulting in the conversion of unmethylated cytosine residues to uracil. Methylated cytosine residues are protected from this reaction. Two sets of polymerase chain reaction (PCR) primers were used. The first set was specific for a DNA sequence containing methylated cytosines. The second set was specific for the analogous DNA sequence containing unmethylated cytosines. The primers used for the methylated reaction were sense-5'TTTCGACGTTCGTAGGTTTTCGC3' and antisense-5'GCACTCTTCCGAAAACGAAACG3'. The primers used for the unmethylated reaction were sense-5'TTTGTGTTTTGATGTTTGTAGGTTTTTGT3' and antisense-5'AACTCCACACTCTTCCAAAAACAAAACA3' (Esteller et al., 2000b). Each 25-μl amplification reaction with methylated template contained the 10× ammonium sulfate reaction buffer described by Herman et al. (1996), 320 nM of primers, 500 μM of deoxynucleoside triphosphate, 2 units of Amplitaq Gold (Perkin-Elmer, Foster City, Calif.), and 100 to 800 ng of bisulfite-modified DNA and H_2O. The amplification reactions with unmethylated template differed in the amount of primer (240 nM), dNTPs (240 μM), and Amplitaq Gold (1 unit). The cycling program employed 96°C for 10 min; then 30 cycles of 96°C for 20 s, 59°C for 20 s, and 72°C for 30 s; followed by 72°C for 4 min. A band of the proper size visualized on polyacrylamide gel electrophoresis from the primers specific for the methylated sequence indicates methylation of cytosine residues in the primer binding sequence (Fig.1). DNA from the cell line SW48, which is known to have a methylated *MGMT* gene, was used as a positive control (Esteller et al., 2000b). No-template reactions as well as reactions containing bisulfite-treated DNA from normal lymphocytes were used as negative controls.

MS-PCR Product Restriction

The amplicon of the above primers contains 11 CpG dinucleotides, 7 of which are contained in the primer binding sites. An additional 2 CpG dinucleotides were tested for methylation by cleaving the PCR product with the restriction endonuclease *BstUI* (New England Biolabs, Beverly, Mass.), which recognizes the sequence CG/CG. Unmethylated cytosine residues in the recognition sequence are converted to thymidine by bisulfite treatment followed by PCR amplification, abrogating *BstUI* cleavage. The restriction endonuclease *HphI* (New England Biolabs) recognizes the sequence GGTGA, which is created by bisulfite treatment of the unmethylated template GGCGA. Therefore, *HphI* was used to confirm the methylation status of the unmethylated MS-PCR products (Fig. 1). PCR products were incubated in the supplied buffer according to the manufacturer's recommendations, and the restriction products were run on a 9% nondenaturing polyacrylamide gel, stained with ethidium bromide and visualized under UV light.

Quantitative Real-Time Reverse Transcriptase PCR

We used Trizol (Invitrogen, Carlsbad, Calif.) to extract RNA from 19 samples of pediatric medulloblastoma that had been taken from the center of the tumor mass and intraoperatively flash-frozen in liquid nitrogen. Complementary DNA (cDNA) was created from total RNA by using the Reverse Transcription System kit (Promega, Madison, Wisc.) and oligo-dt primers. cDNA was amplified in the presence of SYBR green (Biowhittaker, Rockland, Maine) by using primers specific for the housekeeping gene β-*actin* (sense-5'GCCATGCCAATCTCATCTT3' and antisense-5'ACCTGTACGCCAACACAGTG3') and MGMT (sense-5'CACCGTTTGCGACTTGGTACTT3' and antisense-5'AGACCCTGCTCACAACCAGACA3'). The MGMT quantitative real-time reverse transcriptase–

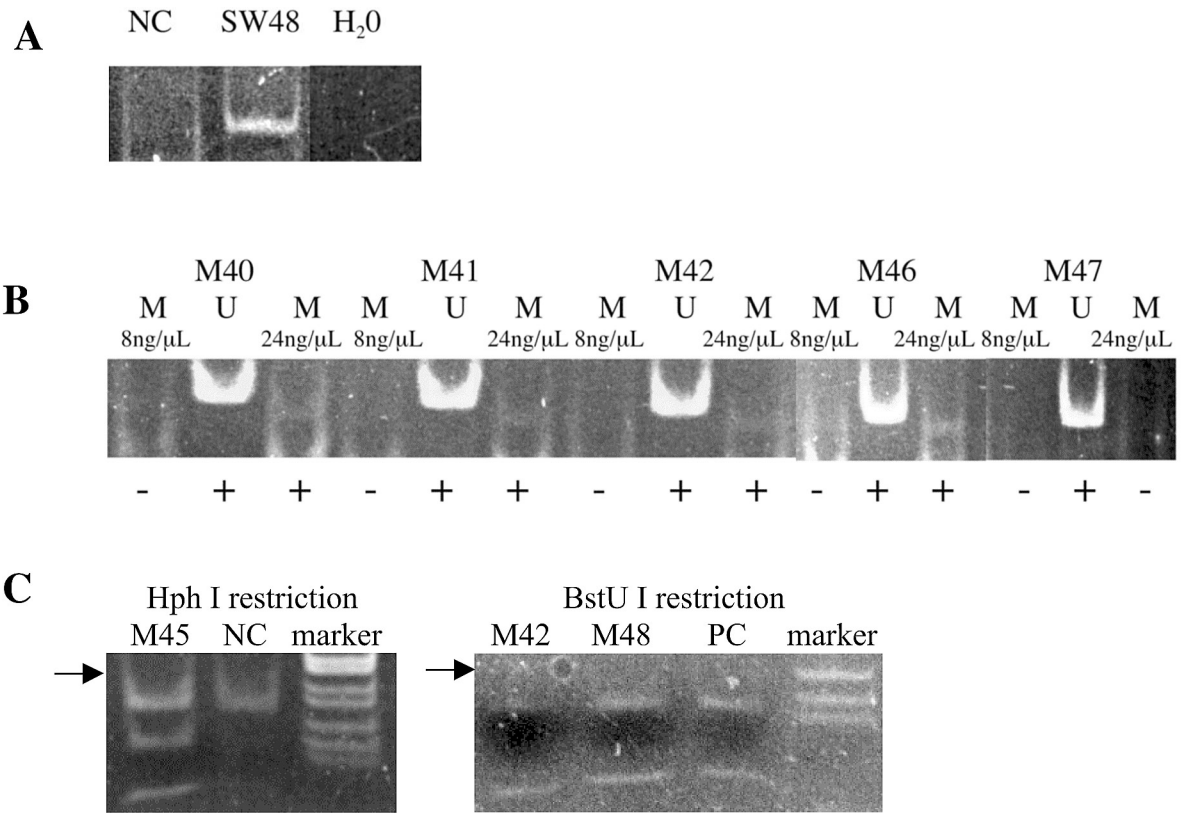

Fig. 1. *A*. Controls used for MS-PCR reaction targeting methylated alleles of the *MGMT* gene include normal lymphocytes for negative control (NC), SW48 (positive control colon cancer cell line), and no template negative controls (H_2O). *B*. Representative polyacrylamide gels of MS-PCR reactions. U denotes reactions using primers specific for unmethylated alleles and M denotes reactions specific for methylated alleles. The methylated reactions are labeled with the concentration of bisulfite-treated DNA template used. *C*. Digestion of MS-PCR products with either *Hph I* to confirm an unmethylated internal cytosine or *BstU I* to confirm methylated internal cytosines. NC is negative control and PC is positive control. The arrows indicate the size of the products prior to restriction, and the marker shown is pBR322 DNA-Msp I Digest (New England BioLabs, Beverly, Mass.).

polymerase chain reaction (qRT-PCR) product corresponds to bp 69-179 of GenBank accession number M29971 (NIH, 2003). The cycling program employed 96˚C for 10 min; then 40 cycles of 96˚C for 10 s, 58˚C for 10 s, and 72˚C for 20 s; followed by 72˚C for 4 min. MGMT expression was normalized to β-actin expression and calculated from the threshold cycle by using the iCycler iQ qRT-PCR system (Bio-Rad, Hercules, Calif.).

Immunohistochemistry

Paraffin sections (4 µm thick) were deparaffinized in 3 changes of xylenes over a period of 2 h. The sections were washed 3 times in 100% ethanol and 3 times in 95% ethanol, followed by rehydration in running dH_2O and a 40-min wash in dH_2O. The slides were placed in a container and covered with Vector antigen unmasking solution (Vector, Burlingame, Calif.) and heated at 95˚C for 5 min. The slides were allowed to cool in the buffer for approximately 20 min. They were then washed in deionized H_2O 3 times for 2 min each. Excess liquid was aspirated from the slides, which were then incubated in 1.5% H_2O_2 in H_2O to block endogenous peroxidase. The slides were washed in running dH_2O and 2 changes of phosphate-buffered saline (PBS) and Tween 20 (0.05%) for 5 min each. The slides were then incubated at room temperature in a moisture chamber for 30 min with 5% normal horse serum. After excess serum was removed, the slides were incubated overnight at 4˚C with anti-MGMT antibody (O^6-methylguanine-DNA methyltransferase clone mT3.1 [5 µg/ml]; Chemicon International, Temecula, Calif.). The next morning, the slides were allowed to acclimate to room temperature for 1 h, followed by two PBS/Tween 20 washes of 2 min each. The biotinylated polyclonal secondary antibody was then applied for 30 to 60 min and washed twice for 2 min each in PBS. Subsequently, the peroxidase-conjugated streptavidin was applied for 30 min and washed off with two 2-min washes in PBS. The labeled antibody was localized with DAB solution (1.50 µm 3,3'-diaminobenzidine [Sigma No. D-5637] and 0.1% H_2O_2 dissolved in 0.05 m Tris buffer [pH 7.6]) for 3 min. Counterstaining was achieved with Harris modified hematoxylin (Fisher Scientific, Pittsburgh, Penn.) followed by rapid dH_2O wash. The slides were then dehydrated with 95% ethanol for 30 s, 100% ethanol for 30 s, and xylene for 30 s.

Coverslips were affixed with 1 drop of permanent mounting medium. Normal tonsillar tissue was used as the positive control in each run, and a slide was prepared without the primary antibody to provide the negative control.

Table 1. Minimum concentration of bisulfite-treated template required to demonstrate MS-PCR product for methylated template primers for each tumor sample. There was no correlation between methylation, as measured by MS-PCR, and mRNA expression (1-way parametric ANOVA, $P = 0.61$), nor was there any statistically significant difference between the mean expression values of the groups containing all methylated and unmethylated tumors (Student's *t*-test, 2 tailed $P = 0.52$).

	Bisulfite-Treated DNA Template				
Tumor Sample	>8 ng/μL	>16 ng/μL	>24 ng/μL	No Methylation	*MGMT* Expression*
M30	X				4.8
M55	X				7.2
M56	X				1.4
M57	X				8.2
M58	X				2.6
M62	X				3.2
M63	X				4.0
M69	X				1.0
M44	X				
M48	X				
M50	X				
M52	X				
M53	X				
M60	X				
M32		X			
M34		X			2.2
M37		X			1.0
M45		X			5.4
M59		X			4.2
M61		X			2.0
M36		X			
M38		X			
M40		X			
M41		X			
M46		X			
M49		X			
M42			X		20.6
M35			X		
M31				X	1.4
M33				X	3.0
M47				X	7.4
M64				X	3.0
M66				X	7.6
M28				X	
M29				X	
M39				X	
M54				X	

* *MGMT* expression, as a fold change over the tumor with the least mRNA expression, is noted for each sample tested.

Statistics

To determine if any correlation exists between the methylation status and expression of the *MGMT* gene, we used the Student *t*-test to compare the mean mRNA expression values of the methylated and unmethylated groups. In order to extend this analysis to detect differences in expression between tumors grouped by minimum template concentration necessary to demonstrate methylation, a one-way ANOVA was performed. A Tukey-Kramer multiple comparison test was used to determine if any significant differences exist between any pair of tumor groups. The expression value for M42 (20.6) was discarded as an outlier for the purposes of statistical testing.

Results

MGMT *Methylation*

Thirty-seven medulloblastoma samples obtained from different children were examined for methylation in a region previously identified as a methylation "hot spot" correlated with transcriptional silence of the *MGMT* gene (Costello et al., 1994; Danam et al., 1999; Herfarth et al., 1999; Qian and Brent, 1997). MS-PCR primers specific for the unmethylated template amplified a product of the proper size in all tumor samples at template concentrations of 8 ng/μl. In total, the MS-PCR primers specific for the methylated template amplified a properly sized product in 28 of 37 tumors (76%). However, the tumors can be subdivided by the concentration of starting template required to achieve amplification of the methylated sequence. When the MS-PCR reactions were carried out with bisulfite-treated DNA template at a concentration of 8 ng/μl, only 14 showed a product from the methylation-specific primers (Table 1). When the template concentration was increased to 16 ng/μl, 12 more revealed methylation. Two further samples were found to be positive for methylation when the template concentration was increased to 24 ng/μl. The template concentrations required to amplify products from the unmethylated and methylated, template-specific MS-PCR reactions are consistent with methylation of a relative minority of the *MGMT* alleles extracted from the tumor samples (Fig. 1B). In order to corroborate the specificity of the methylated template primers, a subset of MS-PCR products were incubated with the restriction enzyme *BstUI,* which recognizes the CG/CG site present in the product. All were cleaved into appropriately sized restriction products (22 and 59 bp). A subset of products generated with the primers specific for the unmethylated template were incubated with the *HphI* enzyme, which recognizes the sequence GGTGA, present only in the product of the unmethylated specific primers. All were cleaved into restriction products of the predicted sizes, 63 and 30 bp (Fig. 1C).

MGMT *mRNA Expression*

Fresh frozen tumor tissue sufficient in quantity for *MGMT* mRNA expression analysis was available for 19

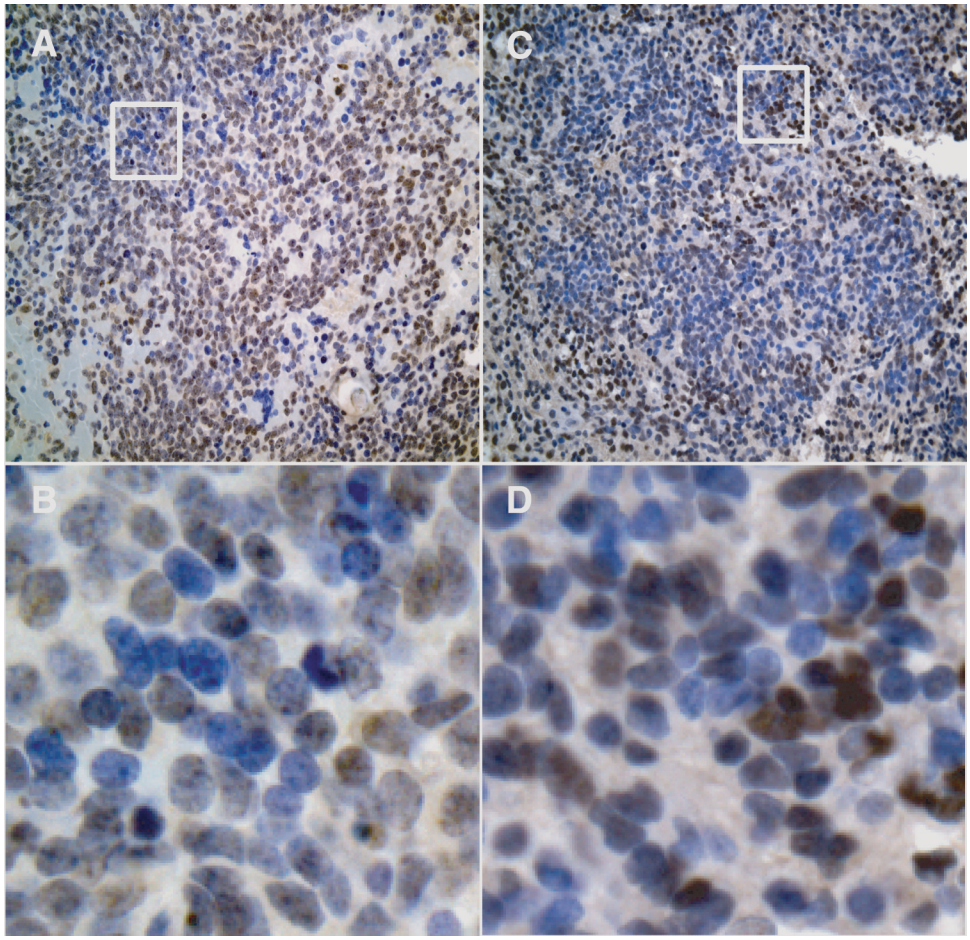

Fig. 2. Representative samples of heterogeneous nuclear staining for MGMT using immunoperoxidase with a hematoxylin counterstain in 2 different medulloblastoma tumor samples. *A*. M48 50×. White box indicates area presented in *B*. *B*. M48 400×. *C*. M58 50×. White box indicates area presented in *D*. *D*. M58 400×.

of the 37 medulloblastoma samples studied with MS-PCR for methylation of the *MGMT* gene. Analysis by qRT-PCR showed expression of *MGMT* RNA in all tumors. Data were normalized to β-actin expression levels and compared to reference cDNA for comparison across tumors. *MGMT* expression, as a ratio to the lowest expressing tumor, ranged from 1 to 20.6 with a median of 3.2, a mean of 4.7, and a standard deviation of 4.5. There was no difference found in the mean *MGMT* expression level between the methylated and unmethylated tumor groups (Student's *t*-test, 2 tailed, $P = 0.52$). There was no correlation found between the minimum template concentration required to demonstrate methylation and the *MGMT* mRNA expression level in this tumor set (1-way parametric ANOVA, $P = 0.61$). The results of these experiments rule out a strong association between *MGMT* methylation and expression in medulloblastoma.

MGMT Protein Expression

Paraffin-embedded tumor tissue for MGMT protein expression analysis was available for 9 of the tumors analyzed for methylation of the *MGMT* gene. All 9 tumors exhibited positive immunoreactive nuclear staining of medulloblastoma cells for MGMT in an intermingled heterogeneous pattern (Fig. 2). Tumor-to-tumor variation in the relative proportions of positive and negative cells was evident. Areas of adjacent normal cerebellar tissue on the sections did not stain for MGMT, while endothelial cells did exhibit positive staining. Focal areas of both positive and negative staining were noted. Positive control tonsillar tissue exhibited uniform positive staining.

Discussion

We have shown that DNA methylation in a region of the *MGMT* gene previously shown to confer transcriptional inactivation does not correlate with decreased total expression of the *MGMT* gene in a panel of pediatric medulloblastoma tumor samples. Our findings also reveal that this discordance can be explained by a heterogeneous pattern of protein expression between individual cells of a medulloblastoma tumor.

DNA methylation, as measured by MS-PCR, has been previously suggested to be a marker of *MGMT* inactivation and MGMT protein deficiency (Esteller et al., 2000b). As such, *MGMT* methylation could be utilized to screen for tumors more likely to be susceptible to methylating alkylator chemotherapy. However, in this study we show discordance between *MGMT* methylation as measured by MS-PCR and *MGMT* mRNA expression in medulloblastoma. Two possible explanations for these results are (1) the presence of contaminating normal cells in the tumor tissue samples and (2) a heterogeneous pattern of *MGMT* methylation among the tumor cells that constitute a given sample. Most previously published studies using MS-PCR for methylation analysis of clinical tumor samples have shown PCR products created by the primers specific for the unmethylated template in tumor samples that also demonstrate methylation. This finding has been explained by the presence of contaminating normal tissue such as infiltrating lymphocytes, reactive astrocytes, or endothelial cells in the tumor specimens (Esteller et al., 1999, 2000b). When DNA is extracted from a tumor sample and bisulfite treated, these contaminating cells, which constitute a small proportion of the cells in a medulloblastoma, contribute only a minor fraction to the total pool of converted template. Therefore, when the converted template is used in MS-PCR, one would expect the unmethylated amplicon to be absent at very low total template concentrations and to begin to appear as the template concentration is increased and the unmethylated contaminating template is raised above the amplification threshold. Results from one half of the tumors showing methylation were inconsistent with this expectation in that unmethylated products were readily demonstrated at the lowest template concentrations (before the methylated products), and methylated products appeared with increasing template concentrations. The remainder of the methylated tumors demonstrated both methylated and unmethylated bands in the lowest template concentration reactions. These results are consistent with the majority of *MGMT* alleles being unmethylated, a greater proportion than would be seen from contaminating normal tissue alone. A heterogeneous pattern of methylation would, however, explain the discordance between *MGMT* methylation and expression and be consistent with the results obtained by increasing concentrations of MS-PCR template.

Heterogeneous methylation and expression of *MGMT* has been demonstrated in brain, colon, and gastric cancers (Bae et al., 2002; Esteller et al., 1999). We performed immunohistochemistry for the MGMT protein on 9 of the medulloblastoma samples and showed a heterogeneous pattern of MGMT protein expression with cell-to-cell variation. These results argue against screening for *MGMT* methylation as a marker of O^6-methylguanine repair deficiency in medulloblastoma. Heterogeneous MGMT expression is consistent with a methylation event occurring after tumorigenesis, giving rise to progenitor cells of differing phenotypes, or a reversible process as has been demonstrated in cell culture (Pieper et al., 1999). Extending this line of reasoning suggests a different role for *MGMT* inactivation from that proposed in colon cancer, where it is permissive of G-to-A transition mutations of the *K-ras* oncogene (Esteller et al., 2000b).

Other potential contributions to the discordance between *MGMT* methylation and MGMT expression include alternative mechanisms of MGMT transcriptional regulation. For example, overexpression of wild-type p53 in cell culture has been shown to result in the transcriptional downregulation of *MGMT*, and this inverse relationship has been demonstrated in clinical samples of infiltrating astrocytoma by immunostaining (Srivenugopal et al., 2001; Yuan et al., 2003). Regional differences in DNA methylation could also play a role. Methylation of discrete hot spots that flank a region containing the minimal promoter, transcription start site, and minimal enhancer has been correlated with *MGMT* gene silencing in studies employing sequencing of bisulfite-treated DNA in expressing and nonexpressing cell lines (Qian and Brent, 1997; Watts et al., 1997). These findings have been confirmed in xenografts and clinical tumor samples (Danam et al., 1999; Herfarth et al., 1999). The relative contribution of the different hot spots to transcriptional repression can not be inferred from the published data. Since the primers used in this study target only one of the hot spots, the possibility exists that epigenetic silencing of the *MGMT* gene could go undetected by the MS-PCR experiments presented here. However, the incidence of aberrant methylation of the hot spot targeted here reaches 80% to 100% (Qian and Brent, 1997; Watts et al., 1997), making this a less likely alternative conclusion. An additional consideration regarding extrapolation of enzymatic activity from the presence of MGMT protein is the potential for functional mutations, a number of which have been identified (Deng et al., 1999; Edara et al., 1999; Kanugula et al., 1995).

Medulloblastoma is the most common malignant brain tumor in children. The propensity for metastasis through the brain and spine has led to the use of craniospinal irradiation (csXRT) as therapy for this tumor. Significant neurocognitive, neuroendocrinologic, and neuropsychologic sequelae result from this approach (Packer et al., 1987, 1989). Current therapeutic strategies using adjuvant chemotherapy attempt reduction of the csXRT dose to ameliorate these side effects while protecting against disease recurrence distant from the primary site of disease. As radiation doses continue to be reduced, reliance upon chemotherapy for regional disease control increases, and its effectiveness must be maximized. The methylating alkylator CCNU is an active agent against medulloblastoma and is part of the most effective known regimen for standard-risk disease (Packer et al., 1994). A potentially effective way to increase the potency of CCNU is the use of the MGMT-inactivating agent O^6-benzylguanine. This drug, which is currently in clinical trials, reduces the cell's ability to repair the cytotoxic DNA damage caused by CCNU without increasing the side effect profile. Tumors for which this strategy could best be applied include those that are MGMT sufficient. This study shows that there is MGMT sufficiency in some proportion of tumor cells in all pediatric medulloblastoma samples analyzed. The use of O^6-benzylguanine in conjunction with standard alkylator-based chemother-

apy may be a rational strategy to intensify chemotherapy, allowing further reduction of the dose of irradiation delivered to the craniospinal axis. This approach may help to diminish the sequelae of csXRT while providing adequate protection against relapse distant from the primary site of disease and emergence of alkylator-resistant disease.

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References

Bae, S.I., Lee, H.S., Kim, S.H., and Kim, W.H. (2002) Inactivation of O6-methylguanine-DNA methyltransferase by promoter CpG island hypermethylation in gastric cancers. *Br. J. Cancer* **86**, 1888–1892.

Belanich, M., Pastor, M., Randall, T., Guerra, D., Kibitel, J., Alas, L., Li, B., Citron, M., Wasserman, P., White, A., Eyre, H., Jaeckle, K., Schulman, S., Rector, D., Prados, M., Coons, S., Shapiro, W., and Yarosh, D. (1996) Retrospective study of the correlation between the DNA repair protein alkyltransferase and survival of brain tumor patients treated with carmustine. *Cancer Res.* **56**, 783–788.

Bobola, M.S., Berger, M.S., Ellenbogen, R.G., Roberts, T.S., Geyer, J.R., and Silber, J.R. (2001) O6-Methylguanine-DNA methyltransferase in pediatric primary brain tumors: Relation to patient and tumor characteristics. *Clin. Cancer Res.* **7**, 613–619.

Costello, J.F., Futscher, B.W., Tano, K., Graunke, D.M., and Pieper, R.O. (1994) Graded methylation in the promoter and body of the O6-methylguanine DNA methyltransferase (MGMT) gene correlates with MGMT expression in human glioma cells. *J. Biol. Chem.* **269**, 17228–17237.

Danam, R.P., Qian, X.C., Howell, S.R., and Brent, T.P. (1999) Methylation of selected CpGs in the human O6-methylguanine-DNA methyltransferase promoter region as a marker of gene silencing. *Mol. Carcinog.* **24**, 85–89.

Deng, C.J., Xie, D.W., Capasso, H., Zhao, Y.J., Wang, L.D., and Hong, J.Y. (1999) Genetic polymorphism of human O6-alkylguanine-DNA alkyltransferase: Identification of a missense variation in the active site region. *Pharmacogenetics* **9**, 81–87.

Edara, S., Kanugula, S., and Pegg, A.E. (1999) Expression of the inactive C145A mutant human O6-alkylguanine-DNA alkyltransferase in E. coli increases cell killing and mutations by N-methyl-N′-nitro-N-nitrosoguanidine. *Carcinogenesis* **20**, 103–108.

Esteller, M., Hamilton, S.R., Burger, P.C., Baylin, S.B., and Herman, J.G. (1999) Inactivation of the DNA repair gene O6-methylguanine-DNA methyltransferase by promoter hypermethylation is a common event in primary human neoplasia. *Cancer Res.* **59**, 793–797.

Esteller, M., Garcia-Foncillas, J., Andion, E., Goodman, S.N., Hidalgo, O.F., Vanaclocha, V., Baylin, S.B., and Herman, J.G. (2000a) Inactivation of the DNA-repair gene MGMT and the clinical response of gliomas to alkylating agents. *N. Engl. J. Med.* **343**, 1350–1354.

Esteller, M., Toyota, M., Sanchez-Cespedes, M., Capella, G., Peinado, M.A., Watkins, D.N., Issa, J.P., Sidransky, D., Baylin, S.B., and Herman, J.G. (2000b) Inactivation of the DNA repair gene O6-methylguanine-DNA methyltransferase by promoter hypermethylation is associated with G to A mutations in K-ras in colorectal tumorigenesis. *Cancer Res.* **60**, 2368–2371.

Fornace, A.J., Jr., Papathanasiou, M.A., Hollander, M.C., and Yarosh, D.B. (1990) Expression of the O6-methylguanine-DNA methyltransferase gene MGMT in MER+ and MER- human tumor cells. *Cancer Res.* **50**, 7908–7911.

Frommer, M., McDonald, L.E., Millar, D.S., Collis, C.M., Watt, F., Grigg, G.W., Molloy, P.L., and Paul, C.L. (1992) A genomic sequencing protocol that yields a positive display of 5-methylcytosine residues in individual DNA strands. *Proc. Natl. Acad. Sci. USA* **89**, 1827–1831.

He, X.M., Ostrowski, L.E., von Wronski, M.A., Friedman, H.S., Wikstrand, C.J., Bigner, S.H., Rasheed, A., Batra, S.K., Mitra, S., Brent, T.P., and Bigner, D.D. (1992) Expression of O6-methylguanine-DNA methyltransferase in six human medulloblastoma cell lines. *Cancer Res.* **52**, 1144–1148.

Herfarth, K.K., Brent, T.P., Danam, R.P., Remack, J.S., Kodner, I.J., Wells, S.A., Jr., and Goodfellow, P.J. (1999) A specific CpG methylation pattern of the MGMT promoter region associated with reduced MGMT expression in primary colorectal cancers. *Mol. Carcinog.* **24**, 90–98.

Herman, J.G., Graff, J.R., Myohanen, S., Nelkin, B.D., and Baylin, S.B. (1996) Methylation-specific PCR: A novel PCR assay for methylation status of CpG islands. *Proc. Natl. Acad. Sci. USA* **93**, 9821–9826.

Hongeng, S., Brent, T.P., Sanford, R.A., Li, H., Kun, L.E., and Heideman, R.L. (1997) O6-Methylguanine-DNA methyltransferase protein levels in pediatric brain tumors. *Clin. Cancer Res.* **3**, 2459–2463.

Jaeckle, K.A., Eyre, H.J., Townsend, J.J., Schulman, S., Knudson, H.M., Belanich, M., Yarosh, D.B., Bearman, S.I., Giroux, D.J., and Schold, S.C. (1998) Correlation of tumor O6 methylguanine-DNA methyltransferase levels with survival of malignant astrocytoma patients treated with bischloroethylnitrosourea: A Southwest Oncology Group study. *J. Clin. Oncol.* **16**, 3310–3315.

Kanugula, S., Goodtzova, K., Edara, S., and Pegg, A.E. (1995) Alteration of arginine-128 to alanine abolishes the ability of human O6-alkylguanine-DNA alkyltransferase to repair methylated DNA but has no effect on its reaction with O6-benzylguanine. *Biochemistry* **34**, 7113–7119.

NIH. National Institutes of Health (2003) GenBank. National Center for Biotechnology Information (available at http://www.ncbi.nlm.nih.gov/Genbank).

Packer, R.J., Meadows, A.T., Rorke, L.B., Goldwein, J.L., and D'Angio, G. (1987) Long-term sequelae of cancer treatment on the central nervous system in childhood. *Med. Pediatr. Oncol.* **15**, 241–253.

Packer, R.J., Sutton, L.N., Atkins, T.E., Radcliffe, J., Bunin, G.R., D'Angio, G., Siegel, K.R., and Schut, L. (1989) A prospective study of cognitive function in children receiving whole-brain radiotherapy and chemotherapy: 2-year results. *J. Neurosurg.* **70**, 707–713.

Packer, R.J., Sutton, L.N., Elterman, R., Lange, B., Goldwein, J., Nicholson, H.S., Mulne, L., Boyett, J., D'Angio, G., Wechsler-Jentzsch, K., Reaman, G., Cohen, B.H., Bruce, D.A., Rorke, L.B., Molloy, P., Ryan, J., Lafond, D., Evans, A.E., and Schut, L. (1994) Outcome for children with medulloblastoma treated with radiation and cisplatin, CCNU, and vincristine chemotherapy. *J. Neurosurg.* **81**, 690–698.

Pieper, R.O., Lester, K.A., and Fanton, C.P. (1999) Confluence-induced alterations in CpG island methylation in cultured normal human fibroblasts. *Nucleic Acids Res.* **27**, 3229–3235.

Qian, X.C., and Brent, T.P. (1997) Methylation hot spots in the 5′ flanking region denote silencing of the O6-methylguanine-DNA methyltransferase gene. *Cancer Res.* **57**, 3672–3677.

Silber, J.R., Mueller, B.A., Ewers, T.G., and Berger, M.S. (1993) Comparison of O6-methylguanine-DNA methyltransferase activity in brain tumors and adjacent normal brain. *Cancer Res.* **53**, 3416–3420.

Silber, J.R., Bobola, M.S., Ghatan, S., Blank, A., Kolstoe, D.D., and Berger, M.S. (1998) O6-methylguanine-DNA methyltransferase activity in adult gliomas: Relation to patient and tumor characteristics. *Cancer Res.* **58**, 1068–1073.

Srivenugopal, K.S., Shou, J., Mullapudi, S.R., Lang, F.F., Jr., Rao, J.S., and Ali-Osman, F. (2001) Enforced expression of wild-type p53 curtails the transcription of the O(6)-methylguanine-DNA methyltransferase gene in human tumor cells and enhances their sensitivity to alkylating agents. *Clin. Cancer Res.* **7**, 1398–1409.

Watts, G.S., Pieper, R.O., Costello, J.F., Peng, Y.M., Dalton, W.S., and Futscher, B.W. (1997) Methylation of discrete regions of the O6-methylguanine DNA methyltransferase (MGMT) CpG island is associated with heterochromatinization of the MGMT transcription start site and silencing of the gene. *Mol. Cell Biol.* **17**, 5612–5619.

Yuan, Q., Matsumoto, K., Nakabeppu, Y., and Iwaki, T. (2003) A comparative immunohistochemistry of O6-methylguanine-DNA methyltransferase and p53 in diffusely infiltrating astrocytomas. *Neuropathology* **23**, 203–209.