**O**<sup>6</sup>-Methylguanine-DNA Methyltransferase Protein Levels in Pediatric Brain Tumors<sup>1</sup>

Suradej Hongeng, Thomas P. Brent, Robert A. Sanford, Hao Li, Larry E. Kun, and Richard L. Heideman<sup>2</sup>

Departments of Hematology-Oncology [S. H., R. L. H.], Molecular Pharmacology [T. P. B.], Radiation Oncology [L. E. K.], and Biostatistics [H. L.], St. Jude Children’s Research Hospital, Memphis, Tennessee 38105; and Neurosurgery Division, Department of Surgery, LeBonheur Children’s Hospital, University of Tennessee, Memphis, Tennessee [R. A. S.]

**ABSTRACT**

Chloroethylnitrosoureas (CENUs) are commonly used in the treatment of pediatric and adult central nervous system (CNS) tumors. The antitumor activity of CENUs has been hypothesized to be due to an alkylation occurring at the O<sup>6</sup>-position of guanine in DNA. The DNA repair protein O<sup>6</sup>-methylguanine-DNA methyltransferase (MGMT) is responsible for the repair of these potentially cytotoxic lesions and may underlie tumor resistance to CENUs. The current study is the largest report of MGMT levels among newly diagnosed pediatric CNS tumors and the only study that has quantitated MGMT by both biochemical and Western immunoblot assays. Our results show a good correlation between the two methods (r = 0.66). Medulloblastoma/primitive neuroectodermal tumor and ependymoma had the highest level of MGMT, followed by high-grade glioma and low-grade glioma. These data may provide a guide to the use of CENUs in the treatment of pediatric CNS tumors.

**INTRODUCTION**

CENUs<sup>3</sup>, including lomustine (CCNU) and carmustine [1,3-bis(2-chloroethyl)-1-nitrosourea], are bifunctional alkylating agents that are commonly used in the treatment of malignant brain tumors (1). The often poor response of these tumors to such therapy has been hypothesized to be the result of MGMT, a DNA repair enzyme (2-4). MGMT can transfer mutagenic and carcinogenic adducts from the O<sup>6</sup>-position of guanine, and it incidentally repairs precursors of cytotoxic DNA cross-links induced by the CENUs (5, 6). Although there have been several studies outlining the presence and potential role of MGMT in adult malignant gliomas (7, 8), there has been no systematic investigation of MGMT among pediatric CNS tumors, except for limited studies conducted in cell lines or as a small part of a larger survey of adult and pediatric patients (9, 10). Unlike in adults, malignant gliomas make up only a minority of pediatric CNS tumors. More common are embryonal CNS tumors, such as MBs/PNETs and ependymal CNS tumors, which together constitute about 50% of childhood CNS tumors; these neoplasms are rare in adults (11). Here, we report the MGMT content, as measured by both biochemical assay and Western blot immunoassay, in a large group of newly diagnosed and prospectively collected childhood CNS tumors. The data obtained not only provide a comparison of the standard biochemical assay and Western blot immunoassay for MGMT but also are potentially relevant to use of CENUs in the treatment of pediatric brain tumors.

**MATERIALS AND METHODS**

**Patient Population and Specimen Procurement**

Between 1990 and 1995, tumor samples were obtained from 60 patients with newly diagnosed CNS neoplasms at St. Jude Children’s Research Hospital. Patients were between 0.1 and 19.3 years old (median, 5 years) at diagnosis; 33 were male, and 27 were female. Samples were obtained at the time of each patient’s initial definitive surgery and were kept on wet ice in saline-soaked gauze before being snap frozen at −70°C, generally within 2 h of collection. Patients were selected for tissue procurement only by the availability of fresh tissue in sufficient quantity to ensure both MGMT quantification and histological interpretation. Samples were chosen as being representative of the tumor and essentially free of normal brain or necrotic debris.

**Cells and Tissues**

CEM cells (CEM-CCRF line, originally obtained from A. Fridland, St. Jude Children’s Research Hospital) were used as a control; cells were grown in Eagle’s MEM supplemented with 10% newborn calf serum. Prior to preparation of extracts, cells were centrifuged at low speed and washed twice with PBS, and the pellet was quick-frozen in liquid nitrogen and stored at −70°C.

**Extract Preparation**

Frozen tumor samples were weighed before being ground under liquid nitrogen with a mortar and pestle. The powdered tissue was thawed by addition of 2–4 volumes of TEDN buffer [10 mM Tris-HCl (pH 7.5), with 0.1 M NaCl, 2 mM EDTA, 1 mM...
DTT, 0.02% sodium azide, 0.2 mM phenylmethylsulfonyl fluoride, aprotonin (20 trypsin-inhibitor units/ml), and leupeptin (20 μg/ml); Chemicon, Temecula, CA) and disrupted with three 15-s bursts of sonication followed by centrifugation at 98,000 × g (30,000 rpm) in a Beckman Ty 65 rotor for 30 min. The supernatant was removed, quick-frozen and stored at −70°C. CEM cell pellets were thawed in 2 volumes of TEDN disrupted by freeze-thawing three times. After high-speed centrifugation, the supernatant was quick-frozen in liquid nitrogen and stored at −70°C.

Methods of Quantitation

Chemicals. Unless otherwise specified, chemicals were of molecular biology grade and were purchased from Sigma Chemical Co. (St. Louis, MO).

Total Protein Assay. Protein concentration in cell and tissue extracts was determined by the Bradford method (12) using Bio-Rad Protein Assay reagent (Bio-Rad Laboratories, Richmond, CA). BSA was used as the standard.

MGMT Biochemical Assay. MGMT activity in cell and tissue extracts was assayed using [3H]methyl-2,3-nitrosourea-treated calf thymus DNA (13) as a substrate, as described previously (14). Briefly, substrate DNA containing 0.1 pmol of [3H]O6-methylguanine was incubated at 37°C for 30 min with the sample in a final volume of 210 μl of TEDG buffer [50 mM Tris-HCl (pH 7.5), 2 mM EDTA, 1 mM DTT, and 10% glycerol]. The reaction was stopped by addition of 3 ml of 5% trichloroacetic acid, and excess DNA was hydrolyzed by heating it at 80°C for 30 min. Radiolabeled protein was recovered by filtration through Whatman GF/F filters. Following two washes with 10 ml of 5% trichloroacetic acid and one with 5 ml of 95% ethanol, filters were transferred to scintillation-counting vials, before addition of NCS tissue solubilizer (Amersham Corp.) and nonaqueous scintillation fluid for scintillation spectrometry. MGMT activity was calculated as fmol of [3H]methyl transferred from DNA to protein. The levels in brain tumor samples were calculated relative to MGMT in an equivalent amount of extract protein from CEM cells, and after normalizing to MGMT level in CEM cells (~1 fmol/μg) levels are expressed as fmol MGMT/μg extract protein.

MGMT Western Blot Immunoblot. Proteins were separated by electrophoresis in a Bio-Rad (Richmond, CA) minigel apparatus at 200 V for 45 min on 0.75 mm 9% SDS-PAGE slab gels according to the method of Laemmli (15) as described previously (16). Gels were calibrated with Bio-Rad low molecular weight standards. Proteins were transferred onto polyvinylidene difluoride membranes (Immobilon-P; Millipore, Bedford, MA) according to the method of Matsudaia (17) using a Bio-Rad Mini-Trans-Blot cell for 2 h at 140 mA. Blots were blocked with 5% BSA (Immunogold quality; Amersham) and probed with the anti-MGMT mouse monoclonal antibody MT 3.1 (16).

Primary antibody binding was visualized either with gold-labeled secondary antimouse IgG antibody using Auroprobe and Intense SE reagents (Amersham) or by enhanced luminescence using horseradish peroxidase-labeled secondary antibody and ECL reagents (Amersham), in each case following the manufacturer’s instructions. Protein bands in ECL-processed membranes were visualized by exposure to X-ray film (Kodak X-Omat AR, Eastman Kodak Co., Rochester, NY) for time intervals ranging from a few seconds to 30 min.

The intensities of the silver-enhanced gold-stained bands were quantitated by densitometry of bands in transparencies prepared by direct positive photography, and by whole-band analysis on a Bio-Image Visage 110 analytical imaging instrument (Millipore). Bands in ECL-generated X-ray film were quantitated in the same way. Results obtained by the two Western blot immunoblot procedures were essentially identical.

Each set of analyses for brain tumor samples was accompanied by a set of CEM extracts (2–10 μg of protein) containing 4–20 fmol of MGMT as calibration standards. Intensities of brain tumor MGMT bands were calculated relative to the band intensity of equivalent amounts of CEM extract protein, and after normalizing to CEM cell MGMT levels (~1 fmol/μg), tumor MGMT levels were expressed as fmol MGMT/μg extract protein.

Statistical Methods

The association between the MGMT levels obtained with each of the two assay methods was evaluated using the Spearman rank-order correlation coefficient.

The Kruskal-Wallis test was used to compare the distribution of MGMT levels obtained using the two assay methods among the five tumor groups. Later, the pairwise Wilcoxon rank-sum test was used to look for differences between the tumor groups.

To compare the biochemical and Western immunoblot assay levels of MGMT versus patient age, the Spearman rank-order correlation coefficient was again used. A comparison of MGMT levels versus overall and progression-free survival was examined using univariate Cox regression models.

RESULTS

The histological distribution of brain tumor samples evaluated was nearly identical to that observed in the pediatric CNS tumor population at large. LGGs (n = 25) accounted for 42% of tumor samples, MBs/PNETs (n = 14), for 23%, and HGGs (n = 8) for 13%. EPs (n = 6) and anaplastic EPs (n = 2) were combined into the single category of EP, which collectively accounted for 13% of patient samples. The miscellaneous other tumors included choroid plexus carcinoma (n = 2), craniopharyngioma (n = 1), mixed germ cell tumor (n = 1), and meningioma (n = 1). Among the medulloblastoma samples, six came from patients with metastatic disease at diagnosis and eight from patients with local tumor only.

The MGMT levels of essentially all samples were determined by both the biochemical assay (n = 58) and the Western blot immunoblot assay (n = 60). The values obtained by the two assay techniques were well correlated, as outlined in Fig. 1 (r = 0.66, and P = 0.0001).

The rank order of median MGMT levels among the individual tumor groups was similar for both methods of quantitation, and in both the range was large; the difference between the lowest and highest value was 100-fold. The highest levels were observed in MBs/PNETs and EPs, followed by the glioblastoma and other tumors. LGGs had the lowest level (Table 1, Fig. 2). Among those patients in the MB/PNET group, there was no
significant relationship between MGMT level and the presence or absence of metastatic disease ($P = 0.35$). There was no significant relationship between age and MGMT levels in all groups combined, whether the cutoff was 2 or 4 years of age at diagnosis ($P > 0.5$).

Because of small numbers of patients in most tumor groups, the correlation between the MGMT levels and PFS and overall survival was examined only among all patients combined and in the MB/PNET and LGG groups; no statistically significant association with either PFS and overall survival was present for any of these groups ($P > 0.5$). A potential limitation to such an analysis is that none of these patients were treated with CENUs or $O^6$-guanine alkylators, such as procarbazine.

**DISCUSSION**

MGMT is ubiquitously present in both prokaryotic and eukaryotic organisms, suggesting an important evolutionary role related to protection against DNA damage from environmentally and metabolically derived alkylating agents (5). Elevated levels of this protein have been found in several human tumor cell lines, such as colon cancer (18), lung cancer (18, 19), ovarian cancer (19), leukemia (20), and brain tumors (9, 19, 21). These findings suggest that MGMT may be potentially important in determining resistance to CENUs and monofunctional methylating agents, such as procarbazine or temozolamide, which produce $O^6$-guanine DNA adducts. Studies in CNS tumor cell lines and xenografts, and clinical correlations in adult CNS tumors, have suggested an inverse relationship between MGMT levels and survival or response to CENUs (4, 7, 8, 22). Although two previous reports have evaluated MGMT in small numbers of pediatric CNS tumors, one measured activity only in tumor-derived cell lines (9), and the other quantitated MGMT (fmol/cell) in a fashion that does not allow direct comparison of their results with this or other published studies (10). The current report is the largest study of MGMT in pediatric CNS tumors and the first to quantitate levels using separate and confirmatory methods.

The quantitation of MGMT has relied typically on a biochemical assay based on the transfer of the radioactivity from methylated (methyl-3H) DNA to cellular extracts of the protein, as described here in "Materials and Methods." The recent availability of monoclonal antibodies to MGMT has allowed an assay based on antibody probing and identification of MGMT in Western blots of cellular protein (21, 23). Both methods of quantitation were used in this study, thus allowing confirmation of the results of one method with a separate determination by the other, as well as providing a comparison of the fidelity and facility of these two methods for measuring MGMT. As shown in Fig. 1 and Table 1, the correlation between these methods is high: there was no instance of a tumor negative for MGMT, and both methods produced similar results. Our experience with these methods suggests that the Western blot immunoassay is more sensitive and requires less patient material: only 1/10 of the tumor material needed for reliable quantitation by the biochemical assay is needed for the Western blot immunoassay. The Western blot immunoassay may thus be the method of choice.

The level of MGMT has been reported to correlate inversely with response to CENU therapy. Hotta et al. (7) showed that in tumors with MGMT levels $>0.2$ fmol/$\mu$g protein, responses to CENU treatment were significantly fewer than among patients whose tumors had lower levels of the protein. Similar results have been reported by Belanich et al. (8). Additionally, a significant relationship between MGMT levels and survival after treatment with procarbazine has been noted in human xenografts (24). Investigations by others have underscored the relationship between MGMT and tumor response by showing that the depletion of the MGMT by pretreatment with $O^6$-benzylguanine or streptozotocin markedly increases the cytotoxicity of CENUs in vitro as well as in xenografts (25–27). Furthermore, retrovirally mediated transfer of either human or bacterial MGMT genes into mammalian hematopoietic stem cells has been shown to confer CENU resistance (28, 29). Among the pediatric tumors evaluated in the current study, median MGMT levels were highest in MB/PNETs and EPs, followed by the HGG and LGG groups. This relationship was the same for both methods of MGMT quantitation. Statistically,
the range of levels within each of the histological groups overlapped broadly, and there was no significant difference among any of the groups, with the exception of the comparison of MB/PNET versus LGG (P = 0.004) using the biochemical method. A similar rank ordering of MGMT among a variety of CNS tumors from both adults and pediatric patients has been reported by Silber et al. (10), using a different method of quantitation.

Most prior information regarding the levels of MGMT in brain tumors has been obtained in adult HGGs, in which levels have generally been measured to be >0.2 fmol/μg protein (7, 21). The median level of MGMT among the pediatric HGGs in the current study, 0.18 fmol/μg protein, does not differ markedly from that in adults. However, the median MGMT levels in MBs/PNETs and EPs, 0.33 and 0.24 fmol/μg protein by the biochemical and Western immunoblot assays, respectively, appear to be higher than those reported in most adult HGGs; these levels are within the range associated with CENU resistance (>0.2 fmol/μg protein). Relevant to MGMT elevation in these tumors are clinical studies of CENU-containing combination chemotherapy regimens in childhood MBs/PNETs (30) and EPs (31), which showed no significant impact of such treatment on survival. Likewise, the use of these regimens in HGG has generally been associated with only marginal improvement in outcome in pediatric patients (7, 8, 32, 33).

In a report on MGMT in five MB/PNET cell lines, He et al. (9) showed levels of 0.05–1.68 fmol/μg protein using the biochemical assay; three of the five had levels greater than 1.36 fmol/μg protein. These levels are in contrast to the range of 0.1–0.5 fmol/μg protein noted in the 14 MB/PNET samples reported here. This suggests the possibility that the selective pressure associated with cell line development may lead to MGMT levels greater than would otherwise be seen in vitro and affirms the necessity of evaluating the levels of this protein in freshly obtained tissues rather than in cell lines.

The levels of MGMT we have observed in MBs/PNETs question the recent enthusiasm for CENUs in the therapy of these diseases. MGMT levels similar to those noted in MBs/PNETs have been associated with clinical resistance to CENUs in other CNS tumors. Although a recently reported adjuvant chemotherapy regimen of CCNU, vincristine, and cisplatin has been associated with a significant increase in PFS among patients with MB/PNET (34), the limited efficacy of single-agent CCNU in this and other CNS tumors and the well-documented efficacy for platinating agents raise a question regarding the contribution of CCNU to the activity of this regimen. The risk-benefit ratio of the CENUs must be weighed carefully; the prominent hematological suppression and hematological stem cell toxicity, late-onset pulmonary fibrosis, and second malignant tumors associated with these agents suggest they should be used with caution (35).

The newly activated North American pediatric cooperative group study in standard-risk MB/PNET may help to document the clinical activity of CCNU, by comparing similar adjuvant chemotherapy regimens differing only by the use of CCNU versus cyclophosphamide. In the interim, studies correlating MGMT levels with outcome in CENU-based trials or exploration of the MGMT-modulating effects of agents such as $O^6$-benzylguanine in MBs/PNETs and other tumors may be of interest.

REFERENCES


O6-Methylguanine-DNA methyltransferase protein levels in pediatric brain tumors.

S Hongeng, T P Brent, R A Sanford, et al.


Updated version  Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/3/12/2459

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://clincancerres.aacrjournals.org/content/3/12/2459. Click on "Request Permissions" which will take you to the Copyright Clearance Center’s (CCC) Rightslink site.