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Retrospective Study of the Correlation between the DNA Repair Protein Alkyltransferase and Survival of Brain Tumor Patients Treated with Carmustine[1]

Michael Belanich, Monica Pastor, Terri Randall, Denise Guerra, Jeannie Kibitel, Lori Alas, Ben Li, Marc Citron, Patricia Wasserman, Agnes White, Harmon Eyre, Kurt Jaeckle, Susan Schulman, Dori Rector, Michael Prados, Stephen Coons, William Shapiro, and Daniel Yarosh[2]

Applied Genetics Inc., Freeport, New York 11520 [M. B., M. Pa., T. R., D. G., J. K., L. A., D. Y.]; Institute of Molecular and Cellular Biology, University of Singapore, 0511 Singapore [B. L.]; Section of Medical Oncology, Department of Hematology/Oncology, Long Island Jewish Medical Center, New Hyde Park, New York 11042 [M. C., P. W., A. W.]; Medical Affairs & Research, American Cancer Society, Atlanta, Georgia 30329 [H. E.]; Department of Neuro-Oncology, University of Texas M. D. Anderson Cancer Center, Houston, Texas 77030 [K. J.]; Huntsman Cancer Institute, The University of Utah, Salt Lake City, Utah [S. S.]; Southwest Oncology Group Statistical Center, Fred Hutchinson Cancer Research Center, Seattle, Washington [D. R.]; Brain Tumor Research Institute, University of California, San Francisco, San Francisco, California [M. Pr.]; and Barrows Neurological Institute, St. Joseph's Hospital, Phoenix, Arizona [S. C., W. S.]

ABSTRACT

We tested the hypothesis that the level of the DNA repair protein O^6-alkylguanine-DNA alkyltransferase in brain tumors was correlated with resistance to carmustine (BCNU) chemotherapy. Alkyltransferase levels in individual cells in sections from 167 primary brain tumors treated with BCNU were quantitated with an immunofluorescence assay using monoclonal antibodies against human alkyltransferase. Patients with high levels of alkyltransferase had shorter time to treatment failure ($P = 0.05$) and death ($P = 0.004$) and a death rate 1.7 times greater than patients with low alkyltransferase levels. Furthermore, the size of the subpopulation of cells with high levels of alkyltransferase was correlated directly with drug resistance. For all tumors the variables most closely correlated with survival, in order of importance, were age, tumor grade, and alkyltransferase levels. For glioblastoma multiforme, survival was more strongly correlated with alkyltransferase levels than with age. These results should encourage prospective studies to evaluate alkyltransferase levels as a method for identifying brain tumor patients with the best likelihood of response to BCNU chemotherapy.

INTRODUCTION

The incidence of primary brain tumors in the United States is about 15,000 new cases per year, and of these about 30% are high-grade tumors including AAs[3] and GBMs (1). In treating these malignant gliomas, adjuvant chemotherapy with BCNU and its congeners produces an increase in survival compared to radiation alone (2), and BCNU has become the frontline drug in chemotherapy of malignant brain tumors.

BCNU acts by releasing a chloroethyldiazonium ion that alkylates several sites in DNA, the most important of which is at the O^6-position of deoxyguanosine. This chloroethyl adduct undergoes an intramolecular circularization and then crosslinks the DNA, producing a lethal lesion (3). Before crosslinking, O^6-alkylguanine is repaired in human cells by the DNA repair protein O^6-alkylguanine-DNA alkyltransferase (EC 2.1.1.63), which transfers the alkyl adduct to a cysteine residue at position 145 of the alkyltransferase peptide chain and thereby becomes inactivated (4, 5). This stoichiometric "suicide" method of repair means that the number of alkyl adducts that can be repaired is limited by the number of alkyltransferase molecules available.

Repair by alkyltransferase is related to human tumor cell resistance to BCNU (6). A group of human tumor cell lines that lack alkyltransferase activity is designated as having the "Mer$^-$" phenotype (for methylation repair minus; Ref. 7), and is hypersensitive to drugs that produce O^6-alkylguanine, including BCNU (8). The free base inhibitor O^6-benzylguanine (NSC 637037) consumes the alkyltransferase activity of the cell (9), and thereby overcomes drug resistance and sensitizes human xenografts to killing by BCNU (10). O^6-benzylguanine is currently in Phase I clinical testing (IND 45789).

The correlation of tumor alkyltransferase levels with patient response has been hampered to date by the requirement in the standard biochemical activity assay for large samples of fresh tumor tissue and the small number of tumor samples available for such analysis in prospective studies. We have recently developed a quantitative immunofluorescence assay for alkyltransferase using monoclonal antibodies against the DNA repair protein (11) and applied it to sections from brain tumors, including those from archived paraffin blocks (12). This allows the retrospective measurement in tumors from patients treated with BCNU for whom the clinical outcome has been recorded. We report here the study of the largest series to date of alkyltransferase in brain tumor patients and the correlation with response to BCNU.

MATERIALS AND METHODS

Patient Population and Response Criteria. Brain tumor sections and corresponding patient data were obtained for 225 patients who received BCNU adjuvant chemotherapy, 167 of which were evaluated. The criteria for inclusion were histologically confirmed primary brain tumor, adjuvant chemotherapy including BCNU, and availability of patient response data as detailed below. Fifty-eight samples were excluded from the study before examination of the clinical data because sections were too thick or too small for processing (19 samples) or background fluorescence obscured analysis (39 samples).

Four research centers cooperated in providing tumor samples. In the case of Southwest Oncology Group and University of California, San Francisco, patients were taken from one arm of a prospective randomized trial (SWOG 9218 and BTRC 8822). In the case of Barrows Neurological Institute and Long Island Jewish Medical Center, patients were selected on the basis of availability of tumor tissue and complete patient response data. For each center, the number of patients evaluated and the standard treatment protocols for the patients were as follows: (*a*) Barrows Neurological Institute, 45 patients received 60 Gy over 7 weeks, delivered to the tumor bed with a 3-cm margin, and 200 mg/m^2 BCNU i.v. every 8 weeks; (*b*) Southwest Oncology Group (SWOG 9218), 64 patients received standard radiotherapy as determined by the local institution (generally 50–60 Gy in 6–7 weeks) and after 4 weeks, BCNU at 80 mg/m^2/day, in 2 or 3 days every 6 weeks, until disease progression or intervening toxicities required termination of therapy; (*c*) University of California, San Francisco (BTRC 8822), 34 patients received a total dose to the

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[2] To whom requests for reprints should be addressed, at Applied Genetics Inc., 205 Buffalo Avenue, Freeport, NY 11520. Phone: (516) 868-9026; Fax: (516) 868-9143.

[3] The abbreviations used are: AA, anaplastic astrocytoma; GBM, glioblastoma multiforme; BCNU, carmustine; DAPI, 4′,6-diamidino-2-phenylindole; SWOG, Southwest Oncology Group; RT, radiation therapy; 6-TG, 6-thioguanine.

tumor of 6000 rads in a period of 6–7 weeks with 30–35 increments of 180–200 rads/day, 5 days a week. During radiotherapy, hydroxyurea was given at 300 mg/m^2 every 6 h every other day. After completion of radiation therapy, 6-TG was given p.o. at 100 mg/m^2 every 6 h for 12 doses, and BCNU was given i.v. at 210 mg/m^2 between 4–6 h after the last dose of 6-thioguanine; (*d*) Long Island Jewish Medical Center, 24 patients generally received radiotherapy of 60 Gy over 5–6 weeks. All received i.v. BCNU at 200–240 mg/m^2 over 6 weeks. The extent of surgical resection in the patient groups was not reported.

Patient response to chemotherapy was evaluated by two clinical parameters. Time-to-failure was the time in months from the date of first treatment with BCNU until the patient reached treatment failure, defined as an increase in tumor mass by computed tomography and magnetic resonance imaging scans and a decline in Karnofsky rating, or death. Time-to-death was the time in months from the initial BCNU treatment to death. In addition to the patient treatment and response data, the centers provided data on patient age, sex, tumor grade, and other drugs administered. In the case of the SWOG data set, alkyltransferase data were reported by Applied Genetics to SWOG before the patient response data were disclosed to Applied Genetics.

Tumor Sections and Pathological Review. The diagnosis for each of the patients was determined by the referring group pathologist. Five slides with one section 2–6 μm thick were prepared from each brain tumor. Of the 167 tumor samples evaluated, 125 were cut from formalin-fixed and paraffin-embedded specimens, and 42 were cut from frozen blocks of tumor embedded in OCT medium. To determine whether tumor pathology differed between patients with the highest and the lowest alkyltransferase levels, one slide from each of 25 randomly selected patients with high alkyltransferase and 25 randomly selected patients with low alkyltransferase was hematoxylin and eosin stained and was scored by histopathology for integrity of the specimen. Virtually all (94%) of the tissue on the slides was tumor tissue, and over one-half of the slides contained only tumor tissue, with no significant difference between the two groups ($P = 0.28$; Pearson χ^2 test). The average amount of necrotic tissue in the sample was 20%, but one-half of the samples had 5% or less, and there was no difference between high and low alkyltransferase ($P = 0.71$). Seventy-two % showed evidence of inflammation, although one-half were mild cases, again with no difference between the groups ($P = 0.62$). Finally, the majority (54%) of infiltrating cells were lymphocytes, with no difference between the groups ($P = 0.39$).

Quantitative Immunofluorescence Microscopy. Two slides from each tumor were stained in parallel, one with antitransferase antibody (sample) and one without this antibody (background). In each staining session, HT29 cells (72,000 molecules/nucleus and 37% outliers; Ref. 7), were spotted onto poly-L-lysine-subbed slides and were included as a positive control. Paraffin sections were dewaxed and rehydrated through an ethanol series, and frozen sections were rehydrated. Both were then fixed with 4% paraformaldehyde. Quantum Simply Cellular Microbeads (Flow Cytometry Standard, Hato Rey, Puerto Rico), used in calibrating the digitized fluorescence intensity (11), were spotted on slides and fixed in parallel. After washing, tissue samples and beads were treated with 0.1% Triton X-100 in PBS, blocked with 5% nonfat milk/PBS, and stained with antialkyltransferase monoclonal antibody 3B8 (37 μg/ml in block for 60 min at 25°) and secondary goat antimouse IgG antibody linked to FITC (Sigma Chemical Co., St. Louis, MO). The specificity of the primary antibody for human alkyltransferase has been confirmed by Western blot (11), by depletion of staining using either the alkyltransferase inhibitor O^6-benzylguanine or by temozolomide treatment (13), and by the linear proportion of antibody staining to the fraction of Mer$^+$ cells in a mixture of Mer$^+$ and Mer$^-$ cells (13). Nuclei were stained with 0.2 μg/ml DAPI for 3 min, and the slides were rinsed and mounted with 50% glycerol and Slowfade antifade reagent (Molecular Probes Inc., Eugene, OR).

Images from the slides of tissues and beads were captured by epifluorescence microscopy using a Nikon Diaphot microscope equipped with Fluorite lenses and with green and blue filter sets (for FITC and DAPI fluorescence, respectively) and digitized using a Star I CCD Camera (Photometrics Ltd., Tucson, AZ) as described previously (11, 13). Final magnification was ×1300. The images were analyzed with the Optimas image analysis software package (Bioscan Inc., Edmonds, WA), as described (11, 13), using a macro that automates data collection and performs all calculations. Briefly, the nuclei were delineated in the DAPI image, the nuclear outline was superimposed on the FITC image, and then the fluorescence intensity and area of each nucleus were calculated. For each sample, data were extracted from approximately 100 nuclei in the sample slide (antialkyltransferase plus secondary antibodies) and from the background slide (secondary antibody alone). The fluorescence intensity of each nucleus was converted to molecules/nucleus using a calibration curve constructed from the bead standards and the area of the nucleus. The reproducibility of the slope of the bead standards has a standard error of 13%, and the fluorescence intensity of the positive control human tumor cell has a standard error of 7%. This method has a limit of detection of 12,000–30,000 molecules/nucleus.

Molecules/nucleus was calculated by comparing the distribution in the sample population to that of the background. If there was no statistically significant difference between the two distributions, alkyltransferase was not detectable, and the sample was scored as zero alkyltransferase. Otherwise, the value for molecules/nucleus was the difference between the means of the two distributions. Fraction of outliers was the fraction of nuclei with fluorescence greater than 2.4 SDs above the background mean. In the background distribution, approximately 1% of the population was greater than 2.4 SDs above the mean. The calculations were performed in duplicate for each sample, and the results were averaged. If the background was unacceptable because of its skewed distribution, a second set of slides was prepared from the tumor block, and if the problem persisted, the sample was declared unusable (39 of 225 slides).

Statistical Analysis. Means are reported in the form mean ± SE, and comparisons between means were made by *t* test (all *P* values are for the two-tailed test). The correlation of each variable with patient time-to-failure and survival was determined in a stepwise manner with the accelerated failure time model using the Weibull distribution, and significance was determined by the Wald test (2L module of the BMDP statistical analysis software, San Diego, CA). Fraction of outliers and molecules/nucleus are not independent variables and were not included simultaneously in the modeling. Survival and failure curves were generated by the Kaplan-Meier (product-limit) method, and groups were compared by log-rank test (1L module of BMDP).

Two methods were used to analyze the correlation between alkyltransferase levels and survival independent of age. The first method used an age adjustment of survival data. Patients were separated into four age groups (<45, 45–54, 55–64, and >64 years), and their survival data were normalized using death rates for these age groups reported by Shapiro (2). The death rates for these four groups were similar to death rates for these groups in our patient population (data not shown). The second method was by stratified log-rank test using the four age strata. Relative death rates were derived from the Mantel-Cox test. Contingency tables were analyzed by the χ^2 test.

RESULTS

Patient Characteristics. This first large-scale test of alkyltransferase as a factor in brain tumor patient survival was designed as a multicenter retrospective trial to include a larger number of patients than was available through a single prospective study. A retrospective study also has the advantage of including complete data on more long-term survivors than are included in a prospective study. However, it has the disadvantage that not all patients were treated uniformly. We have included statistical analyses of the effects of these nonuniform treatment parameters on patient outcome.

Alkyltransferase was measured in 167 brain tumor specimens from patient who received BCNU-based treatment. The characteristics of the patient population are shown in Table 1. The mean age was 50 years (range, 15–75 years), which was close to the mean age of 51.2 years for all brain tumors (1). Most of the 167 tumors were high-grade gliomas, with 99 (59%) GBMs and 47 (28%) AAs. The remaining tumors were oligodendrogliomas, gliosarcomas, low-grade astrocytomas, and undefined gliomas. Nearly all patients received radiation therapy and BCNU, and only 2 patients received BCNU alone. Thirty-three % of patients received additional treatments, which included cisplatin, 6-thioguanine, steroids, or a combination of these treatments.

Measures of Alkyltransferase. Alkyltransferase levels in the tumor biopsies were measured by two parameters: molecules/nucleus, which measures the average alkyltransferase levels in cell nuclei, and

Table 1 *Patient characteristics*

Total patients	167
Age	
Mean ± SEM	50 ± 1.1
Median	52
Sex (No./%)	
Female	58/35%
Male	109/65%
Tumor Grade (No./%)	
GBM	99/59%
AA	47/28%
Other primary brain tumors	21/13%
Treatment Protocol (No./%)	
BCNU alone	2/1%
BCNU + radiation	109/65%
BCNU, radiation + other chemo	48/29%
BCNU, radiation + other therapy	7/4%
Molecules/nucleus	
All tumors	
Mean ± SEM	132,000 ± 15,000
Median	79,000
GBM	
Mean ± SEM	135,000 ± 16,000
Median	73,000
AA	
Mean ± SEM	161,000 ± 41,000
Median	89,000
Fraction of outliers	
All tumors	
Mean ± SEM	0.19 ± 0.01
Median	0.14
GBM	
Mean ± SEM	0.23 ± 0.02
Median	0.18
AA	
Mean ± SEM	0.14 ± 0.02
Median	0.09

fraction of outliers, which measures the fraction of cells with high levels of nuclear alkyltransferase. The mean number of molecules/nucleus was 132,000 ± 15,000, and the mean fraction of outliers for all tumors was 0.19 ± 0.01. The distribution of both measures of alkyltransferase had broad ranges: 0 to 1.4 million molecules/nucleus and 0 to 0.91 fraction of outliers. Twenty-six % of tumors had no measurable alkyltransferase, and 18% had no outliers. GBMs averaged 135,000 ± 16,000 molecules/nucleus, which was not significantly different from the average of 161,000 ± 41,000 molecules/nucleus for AAs. In contrast, the mean fraction of outliers for GBMs (0.23 ± 0.02) was significantly higher ($P = 0.009$) than the mean fraction of outliers for AAs (0.14 ± 0.02).

This difference between GBM and AA alkyltransferase content was also seen in histograms of the population distribution (Fig. 1, *A* and *B*). The distribution of molecules/nucleus was similar for both GBMs and AAs (Fig. 1*A*), but the distribution of outliers for GBMs was skewed toward higher values compared to that for AAs (Fig. 1*B*).

Correlation of Variables. Variables from the patients' records, including age, sex, tumor grade, and both measures of alkyltransferase were examined as covariates with survival, using the accelerated failure time model. In this model, an equation is fit to the clinical data that assigns a coefficient for each variable. Each variable is then systematically analyzed by removing it from the equation and testing the change in fit of the equation. A covariate is statistically significant when its removal significantly alters the fit of the equation to the clinical data. The significant covariates of those evaluated for high-grade tumors, in order of their statistical strength, were age, tumor grade, molecules/nucleus, and fraction of outliers (Table 2). Age and tumor grade are well-known prognostic factors in brain tumor survival (14); we also found that increases in age and tumor grade reduced survival.

The number of molecules/nucleus was a significant negative covariate as a continuous variable ($P = 0.05$), meaning that for each increase in this value, survival declined. When patients were separated into low- and high-alkyltransferase groups, their survival also differed significantly, with low-alkyltransferase patients surviving longer than high-alkyltransferase patients. This difference was most statistically

Table 2 *Covariates with survival*

Covariate	χ^2	*P* value[a]
GBMs and AAs		
Age[b]	40.8	<0.0001
Tumor grade[c]	11.3	0.0001
Alkyltransferase[d] (molecules/nucleus)	8.4	0.004
Fraction of outliers[e]	3.7	0.06
GBMs		
Alkyltransferase (molecules/nucleus)	15.3	<0.0001
Age	5.6	0.018
Fraction of outliers	4.6	0.03

[a] *P* values were from the Wald Test.
[b] Age groups were <45, 45–54, 55–64, and >64 years.
[c] Tumor grade by referring center.
[d] Low alkyltransferase, ≤60,000 molecules/nucleus; and high alkyltransferase, >60,000 molecules per nucleus.
[e] Fraction of outliers and molecules/nucleus were not included in the accelerated failure time models simultaneously.

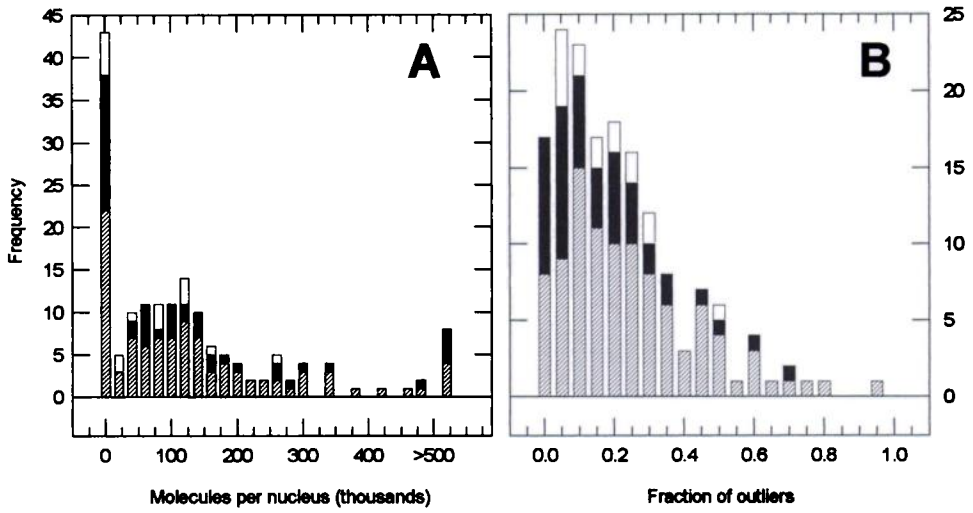

Fig. 1. Distribution of molecules/nucleus (*A*) and fraction of outliers (*B*) for 167 brain tumors analyzed by quantitative immunofluorescence. The histogram was stacked according to tumor type: ▨, GBMs; ■, AAs; □, other primary brain tumors.

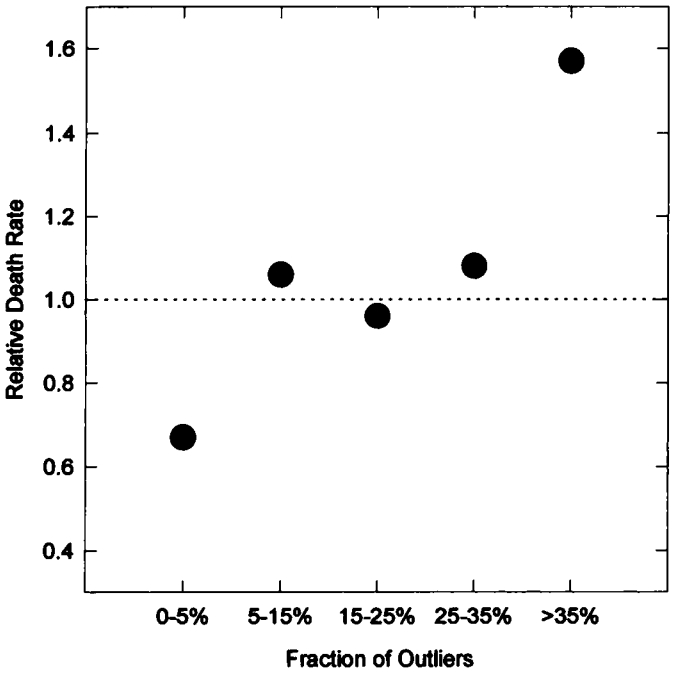

Fig. 2. Correlation of fraction of outliers with death rates. Patients were divided into 5 groups according to fraction of outliers, and the relative death rates were calculated for each group (Mantel-Cox). - - - - -, death rate (1.0) for the overall population.

significant (determined by the maximum χ^2 value) when the groups were divided into those with ≤60,000 molecules/nucleus (low alkyltransferase) and those with >60,000 molecules/nucleus (high alkyltransferase; Table 2). Among the GBM patients, 38% (38 of 99) had low alkyltransferase, and among the AA patients, 49% (23 of 47) had low alkyltransferase, but this difference was not significant ($P = 0.2$). Not only was alkyltransferase level a covariate with survival for all tumors, but considering only the 99 GBM patients, alkyltransferase was the most important covariate with survival, surpassing even age (Table 2). Statistical analysis for the other tumor subtypes was not possible because of low sample number.

Fraction of outliers was a significant negative covariate with survival as a continuous variable (Table 2), and Fig. 2 illustrates this correlation. Patients were grouped according to their fraction of outliers, and the relative death rate for each group was calculated. By definition, the relative death rate for the entire population is 1.0 (dotted line). Patients in the lowest grouping of fraction of outliers had a death rate about one-third lower than the overall group. The relative death rates tended to increase as the fraction of outliers increased, which suggests that as the size of the subpopulation with high alkyltransferase increases, the death rate increases and patient survival declines.

Alkyltransferase and Patient Survival. High- and low-alkyltransferase groups (> or <60,000 molecules/nucleus) were compared by the patient response end points of time-to-failure and time-to-death. Some patients neither had failed treatment nor died at study closure (censored data), and therefore these end points were analyzed by the Kaplan-Meier method and the log-rank test, which accommodate censored data.

Time-to-failure was compared between high- and low-alkyltransferase tumor patients including all tumor grades (Fig. 3*A*) and GBM patients alone (Fig. 3*B*). Patients with low alkyltransferase tumors had a longer time to failure than patients with high alkyltransferase tumors and these differences were significant by log-rank test (all primary brain tumors, $P = 0.05$; GBMs, $P = 0.02$).

Low-alkyltransferase patients also had better survival than high-alkyltransferase patients, not only among all tumor grades (Fig. 4*A*) but also among GBMs (Fig. 4*B*). These results were significant for all tumors and GBMs by log-rank test of age adjusted survival data ($P = 0.004$ and $P = 0.03$, respectively) and by age stratified log-rank test ($P = 0.006$ and $P = 0.03$, respectively). Moreover, there was more than a 2-fold greater percentage of patients with low-alkyltransferase tumors surviving after 1 year than patients with high-alkyltransferase tumors. Considering the survival curves overall, patients with high-alkyltransferase tumors had death rates 1.7 times greater than patient with low alkyltransferase tumors (Table 3).

Nonuniform Treatment. We tested the effect of nonuniform treatment on survival by stratifying the data for the nonuniform treatment variable and applying the stratified log-rank test. When the data were stratified into four groups by treatment center, alkyltransferase was still a statistically significant covariate with survival ($P = 0.0043$; stratified Mantel-Cox test). When the data were stratified into groups by treatment protocol (*e.g.*, BCNU + RT, BCNU + RT + 6-TG, BCNU + RT + steroids, etc.) alkyltransferase remained a statistically significant covariate ($P = 0.0035$; stratified Mantel-Cox test). Another test was to examine age-adjusted 1-year survival within the

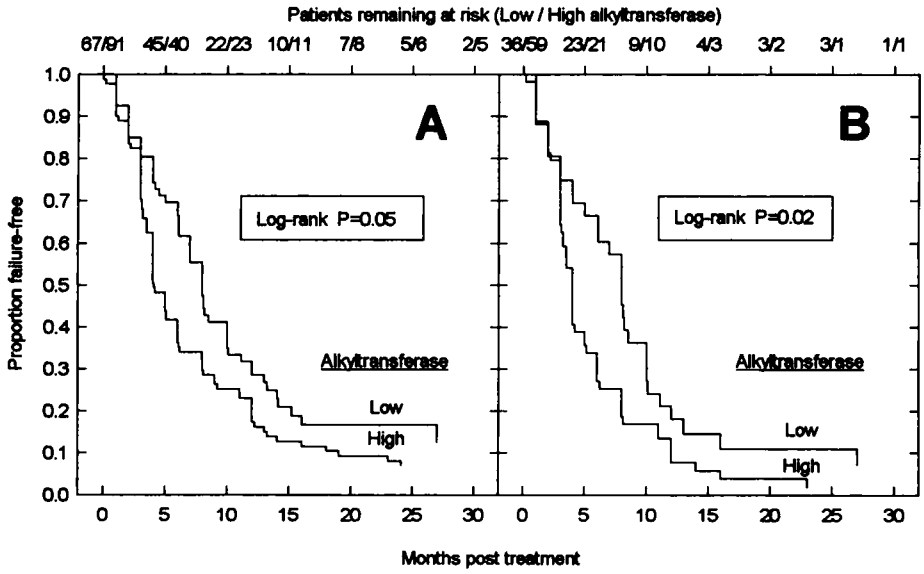

Fig. 3. Patient time to failure grouped by high (>60,000 molecules/nucleus) and low (≤60,000 molecules/nucleus) alkyltransferase. *A*, all primary brain tumors; *B*, GBMs.

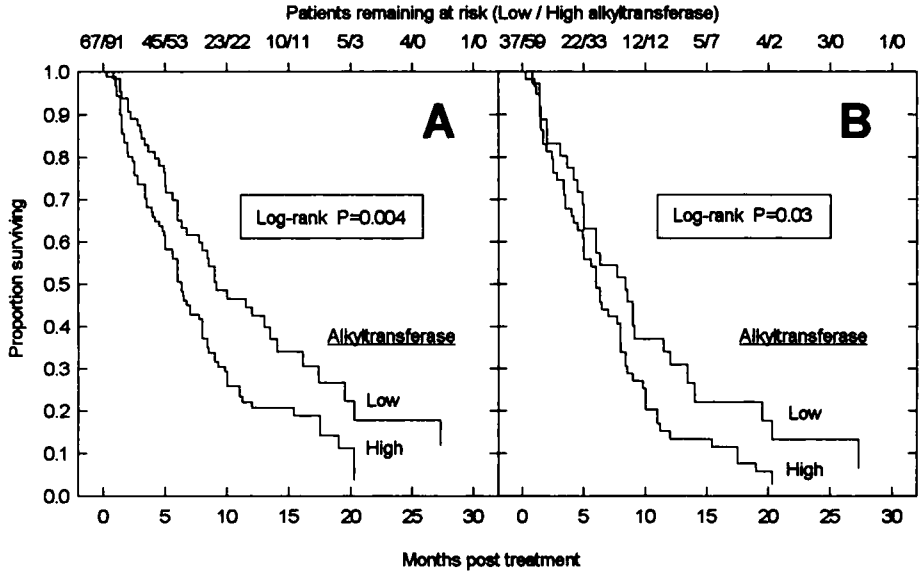

Fig. 4. Patient survival (age adjusted) grouped by high (>60,000 molecules/nucleus) and low (≤60,000 molecules/nucleus) alkyltransferase. *A*, all primary brain tumors; *B*, GBMs.

nonuniform variable using contingency tables. There was no difference in the fraction of patients surviving greater than 1 year either among treatment centers ($P = 0.26$; Pearson χ^2), or between patients receiving BCNU + RT and patients receiving BCNU + RT + other drugs ($P = 0.96$; Pearson χ^2). Therefore, nonuniform treatment of patients could not account for differences in survival observed between patients with high and low alkyltransferase.

DISCUSSION

The capacity for DNA repair by alkyltransferase is remarkably heterogeneous among brain tumors, as has been observed using the biochemical activity assay (12, 15–17) and confirmed in the present series of brain tumor sections by immunofluorescence. In previous biochemical studies, a range of over 10-fold was observed; 15–25% of tumors were completely deficient in alkyltransferase. We found malignant glioma samples with alkyltransferase levels up to 1 log above the mean, and 26% of samples had no measurable alkyltransferase. Differences in the absolute values for alkyltransferase between previous biochemical studies of brain tumors and those reported here arise because of differences in the end point measured (protein activity *versus* antibody-detectable protein) and in the type of sample (fresh tumor extracts *versus* sections from tumor blocks). Of course, biochemical activity measurements cannot be made on retrospective samples from formalin-fixed tumors.

We report here for the first time the correlation between levels of alkyltransferase in human tumors and survival after radiation plus adjuvant chemotherapy with BCNU in a large series of patients. Alkyltransferase correlated with survival as a continuous variable, whether the measure was by molecules/nucleus or by fraction of outliers. Among all tumors, alkyltransferase was a significant covariate ranked behind age and tumor grade, but among GBMs it was the most significant variable.

This multicenter retrospective study requires caution in its interpretation. There was no central pathology or radiographic review for the entire series of patients. In addition, patients were not treated uniformly with radiotherapy or BCNU, many were given additional drugs, and the extent of resection and initial performance status data were not available for all the patients. We examined the effect of these nonuniform variables on survival by stratifying for treatment center or treatment protocol, and found that the correlation between alkyltransferase and poor survival remained statistically significant after either stratification. Furthermore, neither the variable of treatment center nor additional drugs alone accounted for a difference in survival.

The concern of nonuniformity is also addressed by considering only the largest subset of patient data submitted by SWOG.[4] These 64 patients were enrolled and treated uniformly as one arm of a clinical trial (SWOG 9218), all the radiographic and pathology data were centrally reviewed, and the alkyltransferase results were disclosed before patient data were released. Within this group, alkyltransferase was strongly correlated with survival, and the patients with high alkyltransferase had a death rate 2.4 times greater than those with low alkyltransferase ($P = 0.013$; Mantel-Cox log-rank test). This further supports the conclusion that in the overall data set the relationship between alkyltransferase and survival was not an artifact of nonuniform patient treatment.

The average number of alkyltransferase molecules/nucleus was a statistically significant covariate of both time to treatment failure and survival. In addition, by separating patients into low-alkyltransferase (<60,000 molecules/nucleus) and high-alkyltransferase (>60,000 molecules/nucleus) groups, we found a statistically significant difference in both time to treatment failure and survival. Overall, high-alkyltransferase tumor patients treated with BCNU had a death rate 1.7 times that of low-alkyltransferase tumor patients treated similarly. This cutpoint at a relatively low level of alkyltransferase (less than half the mean of 132,000 molecules/nucleus) suggests that even moderate levels of DNA repair capacity confer resistance to alkylating agents. This should serve to emphasize that attempts to sensitize tumors to BCNU chemotherapy by modulating alkyltransferase must achieve profound and prolonged depletion of the repair protein.

The fraction of outliers, or cells within a tumor with significantly elevated levels of alkyltransferase, was also a statistically significant covariate for both time to treatment failure and survival. These out-

Table 3 *Relative rates of response to treatment*

	Alkyltransferase	
Relative rates (Mantel-Cox)	High	Low
Relative failure rate	1.15	0.83
Relative death rate	1.24	0.73

[4] K. Jaeckle, H. Eyre, S. Schulman, D. Rector, M. Belanich, D. Yarosh, *et al.*, manuscript in preparation.

liers are much more common in primary tumor sections than in clonogenic tumor cell lines (13), suggesting they are the product of *in situ* growth conditions. Regional heterogeneity in the DNA of gliomas has been well documented (18, 19), and of particular interest is that chromosome 10, the location of the human alkyltransferase gene (20), is often underrepresented in cells from high-grade tumors (18). Because the fraction of outliers is a continuous variable, fractional increases in this subpopulation reduce the chance for survival. This suggests that resistance to BCNU chemotherapy arises from a subpopulation of drug-resistant cells within the tumor, and that the larger the subpopulation, the larger the fraction of cells that survives BCNU treatment. Such a concept has long been debated and recently supported by model xenograft systems (21), but this report is among the first demonstrations of this principle by clinical correlation of tumor subpopulations with patient response.

Other variables that are prognostic for malignant brain tumors treated with nitrosourea chemotherapy (14) did not obscure the effect of alkyltransferase levels. Age dependence is well recognized and was a significant prognostic factor in this study, but survival differences were still apparent between the high-and low-alkyltransferase groups even after adjustments for age. Tumor grade is also a prognostic variable. GBM tumors have a poorer prognosis than other histological grades, and yet low alkyltransferase tumors also responded better within this group. In fact, for GBMs, alkyltransferase level surpassed age in importance as a prognostic variable.

The demonstration of alkyltransferase as an important covariate of survival in BCNU treatment of brain tumors encourages prospective studies of the prognostic value of alkyltransferase in identifying patients with the best likelihood of response to nitrosourea chemotherapy. This information may assist the oncologist in deciding when aggressive standard therapy is warranted and when alternative therapies might be more fully considered, resulting overall in reduced morbidity and increased patient survival.

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