Quantitative analysis of O^6-alkylguanine-DNA alkyltransferase in malignant glioma

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Abstract

Promoter hypermethylation of the DNA repair protein O^6-alkylguanine-DNA alkyltransferase (AGT) has been associated with an enhanced response to chloroethylating and methylating agents in patients with malignant glioma. The purpose of this study was to compare three distinct yet related indices for measuring AGT to determine if these assays could be used interchangeably when AGT status is to be used to guide chemotherapeutic decisions. Real-time methylation-specific PCR (MSP), assessed as the ratio of methylated AGT copies to internal β-actin control, was used to quantitate AGT hypermethylation in 32 glioma samples. Data were compared with AGT enzyme activity as well as immunohistochemical detection of AGT protein from the same samples. Hypermethylation of the AGT promoter was detected in 19 of 31 (61%) samples evaluable by MSP. Low-level AGT, defined as <20% nuclear AGT staining by immunohistochemistry, was found in 10 of 32 samples (31%), whereas 12 of 32 (38%) had low levels of AGT activity. Correlation of immunohistochemistry to AGT activity was statistically significant ($P = 0.014$) as was the correlation of immunohistochemistry to MSP ($P = 0.043$), whereas MSP compared with AGT activity ($P = 0.246$) was not significant. Cross-tabulation of immunohistochemistry and MSP data based on prognostic groups, where good prognosis was represented by an immunohistochemistry of <20% and an MSP ratio >12, showed no significant relationship ($P = 0.214$), suggesting that one assay cannot be used interchangeably for another. The observed discordance between respective measures of AGT based on prognosis supports further standardization of AGT assays designed to guide therapeutic practice. The data also suggest that consideration be given to the large population of AGT-expressing cells within samples when therapeutic strategies based on tumor methylation are used. [Mol Cancer Ther 2006;5(10):2531–9]

Introduction

The treatment of patients with malignant glioma remains a challenge, with maximal surgical resection followed by focal radiotherapy as the foundation of intervention (1). The addition of systemic chemotherapy, notably with chloroethylating agents such as the nitrosoureas, has been shown to produce a modest increase in survival (1). More recently, the use of the methylating agent, temozolomide, has shown activity in the treatment of recurrent (2, 3) and newly diagnosed malignant glioma (4–6). Unfortunately, but predictably, the benefits of single-agent chemotherapy are substantially reduced by *de novo* or acquired resistance, with consequent tumor progression and patient death.

Accordingly, many laboratory and clinical investigators are actively pursuing drug resistance with the goal of defining, and hopefully modulating, those mechanisms operational in the clinic. These studies have clearly identified the role of O^6-alkylguanine-DNA alkyltransferase (AGT or O^6-methylguanine-DNA methyltransferase) in mediating resistance to chloroethylating and methylating agents such as 1,3-bis(2-chloroethyl)-1-nitrosourea (carmustine) and temozolomide, respectively (7). This protein removes, in a suicide process, the monoadduct generated at the O^6 position of guanine by these agents, thus protecting the cell from antineoplastic activity. AGT is a ubiquitous protein typically present in all normal cells as a defense mechanism against exogenous or endogenous insults. However, the higher levels of AGT frequently seen in malignant glioma compared with normal brain provides a mechanism for the tumor cells to reduce or eliminate the cytotoxic effects of chloroethylating or methylating agents used as antiglioma therapy (8–12).

A preponderance of clinical trials have shown an inverse relationship between low AGT level and positive outcome or high AGT and poor outcome, respectively, in patients with malignant gliomas who are treated with 1,3-bis(2-chloroethyl)-1-nitrosourea (13–21) or temozolomide (4, 22, 23), although a few studies have produced contrary results (24–29). However, these studies have used a spectrum of variables to assess AGT levels in tumor samples, including quantitation of mRNA (15, 19) protein (4, 12, 14, 29), activity (16, 25), and methylation of the promoter (17, 18, 20–23). These various methodologies have not been standardized or, in some cases, validated, precluding

Received 2/24/06; revised 8/8/06; accepted 8/25/06.

Grant support: NIH grants 2RO1 NS-30245-17A1.

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doi:10.1158/1535-7163.MCT-06-0106

universal acceptance of an AGT quantitation which can be used reliably a priori to make therapeutic decisions at the present time.

In this report, we quantitate and correlate three commonly used, yet nonstandardized methods, of measuring AGT in human glioma specimens aliquots and discuss the potential benefits and pitfalls of each assay. Immunohistochemistry was used to quantitate AGT protein expression in formalin-fixed paraffin-embedded histologic sections. AGT activity, defined as the loss of O^6-[^{3}H]methylguanine from an ^{3}H-methylated DNA substrate in fresh-frozen tissue, was measured by high-performance liquid chromatography (HPLC). The methylation status of the AGT promoter region was assessed by real-time methylation-specific PCR (MSP).[8] To our knowledge, this is the first example of a side-by-side comparison of immunohistochemistry, AGT activity, and real-time AGT MSP in clinical tumor samples from patients with newly diagnosed or recurrent malignant glioma.

Materials and Methods

Brain Tumor Samples

Malignant glioma specimens were obtained from patients who had undergone tumor resection at Duke University Hospital. Informed consent was obtained from each patient prior to surgery in accordance with Duke Internal Review Board stipulations. Tissues in excess of that needed for diagnostic pathology were submitted for further analysis of AGT levels. Those tissues for activity analysis and promoter methylation analysis were frozen in Tissue-Tek optimal cutting temperature compound (Sakura Finetek, Torrance, CA) and frozen at −140°C. Slides were cut from these tissue blocks and stained with H&E to confirm the composition/homogeneity of all samples used; tissue blocks used for these experiments were almost 100% tumor cell–positive. A single tissue block was partitioned to perform both AGT MSP and AGT activity assay (described below). Following the rendering of a final diagnosis, a representative block of formalin-fixed paraffin-embedded tumor from each case analyzed above was chosen from the diagnostic archives and histologic sections were cut for immunohistochemistry and AGT localization.

AGT Promoter Methylation Determined by MSP

Genomic DNA was obtained from a 25 mg section of each tissue block using the Puregene DNA purification kit (Gentra Systems, Minneapolis, MN) according to the manufacturer's instructions. DNA was quantified using a PicoGreen assay (Invitrogen, Carlsbad, CA). MSP was done by Oncomethylome Sciences, S.A. (Liege, Belgium).[8] Briefly, 1 μg of DNA was sodium bisulfite–modified using the EZ DNA Methylation Kit (Zymo Research, Orange, CA) according to the manufacturer's instructions. The analyte (mAGT and β-actin) quantitations were done in real-time PCR assays using the ABI Prism 7900HT (Applied Biosystems, Foster City, CA). These consisted of parallel amplification/quantitation processes using the AGT forward primer: 5′-agcgatgcgttcgagcatcgctttcgacgttcgtaggttttcgc-3′, reverse primer: 5′-ctcgaaactaccaccgtcccga-3′. The amplicons created during the amplification process were quantified by real-time measurement of the emitted fluorescence; Amplifluor detection elements are underlined in the AGT forward primer (Chemicon, Temecula, CA). The ratio between methylated AGT and the independent reference gene β-actin was calculated. This ratio was defined as the test result (test result = copies methylated AGT/copies β-actin × 1,000). Oncomethylome Sciences, in collaboration with Hegi et al., did a large scale side-by-side comparison of MSP detection by an OMS assay (described herein) and a gel-based assay[8] used by Hegi in the previously reported EORTC glioma trial (23). This collaboration defined the lack of promoter methylation as a test result <5, whereas a test result of >12 correlates with detectable promoter methylation in the analysis of agarose gel electrophoresis products previously described by Hegi et al. (22, 23, 30). AGT/β-actin ratios between 5 and 12 are considered indeterminate; the correlation of these values to the gel-based assay have not yet been resolved, therefore, these MSP datapoints are excluded in the correlation studies presented herein.

AGT Nuclear Staining by Immunohistochemistry

Immunohistochemistry was done as described previously (4, 31). Briefly, embedded tissue sections were deparaffinized in xylene for 4 hours. Sections were washed in absolute alcohol, blocked in 1.85% H_2O_2/methanol, then rehydrated in deionized water. Antigens were retrieved following heating in AR-10 buffer (Biogenex, San Ramon, CA) for 10 minutes followed by cooling for 30 minutes. Slides were washed twice in PBS, blocked with 5% normal goat serum for 15 minutes, and then incubated overnight with anti-AGT antibody (mT3.1) or control mouse IgG_1 at a concentration identical to mT3.1. Slides were washed twice in PBS, incubated with secondary antibody, and resolved using a multilink horseradish peroxidase detection system developed with 3,3′-diaminobenzidine solution. Slides were counterstained with Harris-modified hematoxylin. Nuclei of 1,000 tumor cells were quantitated independently by two observers to determine the percentage of positive immunoreactive nuclei. The results were accepted if the comparison of the two independent quantitations differed by <5%. Cytoplasmic-only and granular nuclear reactivity were regarded as negative.

AGT Activity Assay

AGT activity was determined as described previously (32, 33). Briefly, tumor tissue was homogenized twice at high speed for 30 seconds using a polytron homogenizer (Glen Mills, Clifton, NJ) in 2 mL/g buffer [50 mmol/L Tris-Cl (pH 7.5), 0.1 mmol/L EDTA, and 5 mmol/L DTT]. Homogenates were sonicated twice (30 seconds) then centrifuged (30 minutes, 4°C, 27,000 × *g*). The protein concentration was determined by the Bradford method (33)

[8] Califice, S., Diserens, A., Straub, J., Vlassenbroeck, I., DiStefano, I., Moreau, F., Klaver, E., Renard, I., Bigley, J., Hegi, M.E., Bierau, K. 2006. A new method for testing MGMT gene promoter methylation status of glioblastoma tissue using a direct real-time fluorescence-based methylation-specific PCR. In preparation.

using bovine serum albumin standard. Calf thymus DNA methylated with ^{3}H-methylnitrosourea (Amersham Biosciences, Piscataway, NJ) was incubated with 1 mg of extracted protein in 0.05 mol/L of Tris-Cl (pH 7.8), 0.008 mol/L of DTT, 10 μg of calf thymus DNA, at 37°C for 30 minutes. The DNA was precipitated in ice-cold perchloric acid (0.25 mol/L) then depurinated in 0.1 mol/L HCl (70°C, 30 minutes). Modified bases were separated by reverse phase HPLC. AGT activity was determined as the loss of O^{6}-[^{3}H]methylguanine from ^{3}H-methylated DNA per milligram of extracted protein, expressed in femtomoles per milligram.

Statistical Analysis

Data were analyzed with S-Plus and SAS. The relationship between immunohistochemistry, AGT activity, and MSP assays for the measurement of AGT was analyzed by Pearson correlation, along with a test of whether the correlation was nonzero. Multiple regression was used to assess the joint effect of age, Karnofsky performance status, tumor grade, and gender on AGT levels measured by immunohistochemistry, AGT activity, or MSP, whereas a two-sample Wilcoxon test was used to determine the independent relationship of each aforementioned predictor with MSP. Fisher's exact and McNemar's tests were used in analyses examining the relationships among dichotomized measures of immunohistochemical and MSP data. κ statistic was also calculated as an alternate measure of agreement of dichotomized data.

Table 1. Patient/tumor characteristics

Characteristics	No. of patients, n = 32 (%)
Age, y	
Median (49)	
Range (20–89)	
Sex	
Male	17 (53)
Female	15 (47)
Histology	
A (WHO grade 2)	1 (3)
XA (WHO grades 2–3)	1 (3)
AO (WHO grade 3)	3 (9)
AA (WHO grade 4)	5 (16)
Glioblastoma multiforme (WHO grade 4)	22 (69)
Karnofsky score	
Median (87)	
Unknown	2 (6)
>70	0 (0)
70–90	25 (78)
100	5 (16)
Chemotherapy	
No treatment	21 (66)
Radiation	0 (0)
Radiation + temozolomide	6 (19)
Temozolomide	1 (3)
Other/combination	4 (12)

Results

Patient and Tumor Characteristics

Tumor specimens from 32 patients (17 men and 15 women) were obtained for this study over a period of 7 months, between June 2004 and February 2005. The median age was 49 years (range, 20–89 years). Twenty-two of the tumors were grade 4 glioblastoma multiforme, with the remainder being grades 2 and 3. Karnofsky performance status was >70% in all cases. The majority of patients received no prior therapy. These data are summarized in Table 1.

Methylation of AGT Promoter Determined by MSP

Thirty-one of the 32 samples were evaluable by MSP. This methodology was devised by Oncomethylome Sciences to introduce an assay using a reference gene to assess the quality of DNA for analyses and an objective cutoff value to remove the subjectivity of reading gel bands. This method has been validated against the more commonly used gel-based MSP assays[8] (17, 22, 23, 30) and is currently being applied in a large, ongoing, prospective clinical trial to stratify glioma patients by AGT methylation status. The findings of this study, testing different schedules of temozolomide, will be used to determine the clinical utility of the assay and better define the optimal cutoff value to differentiate potential clinical outcome differences. The assay used in the current study defined detectable methylation of the AGT promoter as an AGT/β-actin ratio (×1,000) >12. Unmethylated promoters are defined as having an AGT/β-actin ratio <5. Using these cutoff values, promoter methylation of the AGT gene was found in 61% of the tumors and no evidence of gene methylation was found in 29% of the tumors (Table 2). The methylation status of three tumors were in the indeterminate range (in addition to one sample which was unevaluable). The degree of AGT promoter methylation detected was broad, spanning AGT/β-actin ratios of 14 to 814. Using a nonparametric two-sample Wilcoxon test, neither grade (P = 0.999), sex (P = 0.963), Karnofsky performance status ≥90 (P = 0.507), nor age >45 (P = 0.299) were determined to statistically affect MSP outcome (Table 3). Multiple regression analysis was also conducted to determine the relation between these variables and MSP; the outcome was the same.

AGT Protein Expression

AGT protein expression was determined by immunohistochemistry (Table 2). AGT protein expression was detected in 0% to 85% of the cell nuclei. In a previous study, we found that a level of AGT expression, defined as ≥20% AGT-positive cell nuclei, correlated with resistance to temozolomide (4). Using this criteria, 22 of 32 (69%) samples exhibited high AGT protein expression, whereas 31% of tumors expressed low AGT. Low-level AGT protein was observed in both newly diagnosed (6 of 22) and previously treated patient samples (4 of 10). Grade, gender, Karnofsky performance status, and age were not accurate predictors of AGT protein expression using immunohistochemistry as assessed by multiple regression analysis.

Table 2. Comparison of immunohistochemistry, HPLC and MSP measures of AGT in human glioma samples

Patient no.	Gender	Grade*	Immunohistochemistry†	HPLC‡	MSP§
Methylated promoter samples					
1	F	3	0	57 ± 6	814
2	F	4	1	7 ± 8	61
3	M	4	2.5	261 ± 27	470
4	M	4	5.5	50 ± 22	88
5	F	4	10	26 ± 3	90
6	M	3	11	33 ± 47	372
7	F	4	14	21 ± 49	390
8	M	4	20	87 ± 11	161
9	M	4	24	115 ± 48	127
10	M	4	25	40 ± 5	103
11	M	3	30	17 ± 13	376
12	F	4	39	76 ± 59	506
13	F	4	42	0	584
14	F	4	45	0	248
15	M	4	60	133 ± 40	191
16	M	4	60	34 ± 67	268
17	F	4	60	283 ± 20	14
18	M	2	70	248 ± 42	42
19	M	3	70	259 ± 29	91
Unmethylated promoter samples					
20	F	4	5.3	13 ± 14	0.7
21	M	4	24	47 ± 13	0
22	F	4	35	73 ± 37	0
23	M	3	46	22 ± 8	0
24	M	4	51	140 ± 13	0
25	F	3	56	69 ± 1	3
26	M	4	62	238 ± 67	0
27	F	2	65	116 ± 77	0
28	F	4	85	136 ± 1	0
Indeterminate promoter samples					
29	M	3	7	121 ± 9	9
30	F	4	10	16 ± 8	10
31	M	4	66	103 ± 21	n/a
32	F	3	70	60 ± 12	5

*Grade according to WHO I-IV scale.
†Immunohistochemical score, percentage of cells expressing nuclear AGT staining.
‡AGT activity fmol/mg protein.
§Copies methylated AGT promoter/β-actin × 1,000.

AGT Activity

The activity of the AGT protein within tumor samples was quantitated in all 32 tumor samples (Table 2). In three samples, the activity measured was below the limit of detection for this assay (<10 fmol/mg protein). Nine additional tumors had a low level of AGT activity (ranging from 10 to 44 fmol/mg protein), seven had a moderate level of activity (ranging in activity from 45 to 80 fmol/mg protein), and 13 had a high level of activity (>80 fmol/mg).

Correlation of Immunohistochemistry, AGT Activity, and MSP Assays for the Measurement of AGT

To determine the correlation between the data described above, we generated pairwise Pearson correlation coefficients along with a test of whether the correlation was nonzero. The Pearson correlation coefficient for immunohistochemistry versus AGT activity (0.430) suggests a statistically significant ($P = 0.014$) degree of correlation between these measures of AGT, which was also shown by linear regression (Fig. 1A). Comparison of the range of AGT activity in samples with detectable methylated (0–70%) versus unmethylated (5.3–85%) AGT promoters found no significant difference, whereas a slightly lower average immunohistochemical value was observed in the samples with methylated as opposed to unmethylated AGT promoters, 31% versus 48%, respectively. The Pearson correlation coefficient of immunohistochemistry and MSP (−0.385) was also statistically significant ($P = 0.043$), demonstrated by the weak slope showing an inverse relationship by linear regression analysis (Fig. 1B). However, the correlation of MSP to HPLC was not statistically significant ($P = 0.246$; Fig. 1B, *inset*).

We then analyzed the correlation between immunohistochemistry and MSP based on the proposed prognostic values. Good prognosis was suggested by an immunohistochemical

score of <20% (poor prognosis, ≥20%) whereas good prognosis for MSP was defined as an AGT/β-actin ratio >12 (poor prognosis, <5). Eight samples had a good prognosis by immunohistochemistry. Seven shared a good prognosis by MSP, whereas discordance was observed in one sample. Of the 20 samples with a poor prognosis by immunohistochemistry, and evaluable MSP, 8 were gauged as poor prognosis by MSP, whereas discordance was found in 12 samples. Similar data was observed when first analyzing based on methylation status. Nine samples had a poor prognosis by MSP, eight of which had a poor prognosis by immunohistochemistry. Of the 19 samples that were regarded as good prognosis by MSP, 7 were also regarded as good prognosis by immunohistochemistry, whereas discordance was found in 12 samples. Interestingly, in tumors characterized as unmethylated by the MSP assay, the population of AGT protein-negative cells is large, ranging from 15% to 94%, whereas 12 of 19 (63%) methylated samples contain a large population of cells (≥20%) expressing AGT protein. By McNemar's test, there is a statistically significant (P = 0.0023) disagreement between the categorization of patients by immunohistochemistry and MSP based on the assigned good/bad prognosis criteria, suggesting that the outcome of one method could not be used interchangeably for another. In addition, an alternate measure of agreement, the κ statistic, was calculated to be 0.195 with a 95% confidence interval of (−0.051, 0.440). By this methodology, values for κ >0.75 represent excellent agreement, whereas those <0.4 represent poor agreement (34). The calculated κ value and confidence interval are consistent with the conclusion that there is limited evidence that categorized immunohistochemistry and MSP outcomes agree.

Discussion

Chemotherapeutic options for the treatment of malignant glioma remain limited. Nitrosoureas and, more recently, temozolomide produce modest benefits due to the problem of *de novo* and acquired tumor resistance to both classes of agents. The DNA repair protein AGT removes the mono-adduct induced by these agents at the O^6 position of guanine, and is accordingly a major mechanism of resistance (7). AGT depletion using the competitive inhibitor O^6-benzylguanine has been shown to enhance both nitrosourea and temozolomide activity in laboratory (35–37) and clinical investigations (38), consistent with the role of AGT in mediating drug resistance. Recent reports have shown an inverse correlation between AGT measurements and patient response to 1,3-bis(2-chloroethyl)-1-nitrosourea and/or temozolomide drug therapy. Accordingly, there is an increasing interest to use AGT assays as prognostic and therapeutic compasses. This practice is premature as these assays have not been standardized nor validated for clinical use, and the pitfalls and methods of interpreting the results of these assays are not well understood. Therefore, the goal of this article was to examine the correlation between three assays which

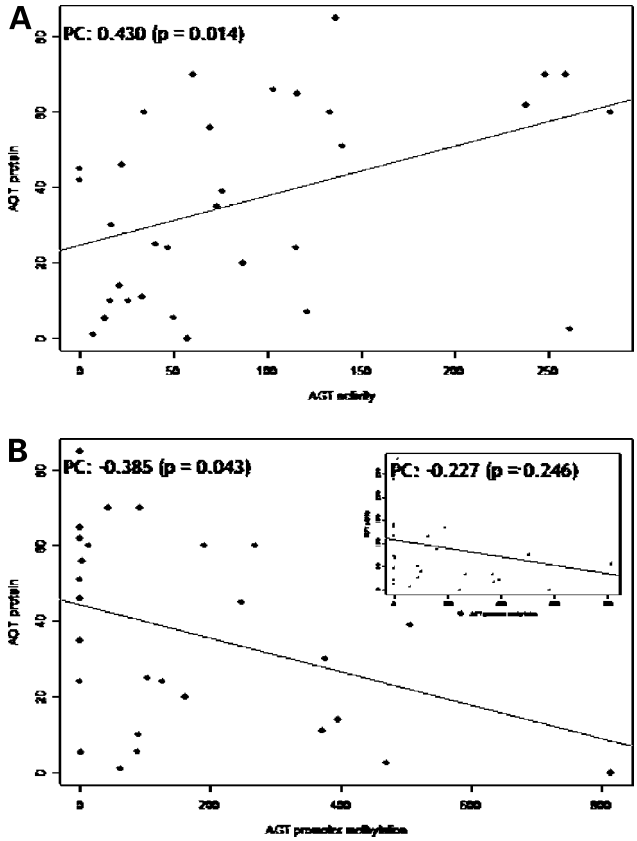

Figure 1. Linear regression analysis of AGT assays. **A,** linear regression analysis of immunohistochemical scored as the percentage of cells expressing AGT protein following staining with AGT-specific antibody (mT3.1) plotted against AGT activity expressed as fmol/mg protein. *Points,* individual tumor samples. **B,** linear regression analysis of AGT protein expression (immunohistochemistry) plotted against AGT promoter methylation determined by MSP, expressed as the ratio of methylated AGT copies to β-actin copies × 1,000. *Inset,* linear regression of AGT activity compared with AGT promoter methylation (MSP). The Pearson correlation (*PC*) coefficients are included along with associated *P* values.

Table 3. Patient characteristics according to AGT methylation pattern

Patient no.	Unmethylated AGT	Methylated AGT
Total no. of patients	9 (29%)	19 (61%)
Sex		
Male	4	11
Female	5	8
Average age	44	53
Grade		
1	0	0
2	1	1
3	2	4
4	6	14
Karnofsky performance status		
Unknown	1	1
70–90	5	16
100	3	2

measure different yet related indices for the quantitation of AGT, and to determine if the assays can be accurately interchanged when defining the significance of AGT in human malignant glioma clinical samples.

Immunohistochemical detection of the AGT protein is the most common and technically least complex method of quantifying AGT. Commercially available antibodies, including clone mT3.1 (39) are produced by >20 companies. Furthermore, many groups continue to develop antibodies directed at differing AGT epitopes with different affinities. Immunohistochemistry has many benefits including the longevity of usable paraffinized samples and small required starting material. In addition, immunohistochemistry has the advantage of allowing tumor cells to be identified from nonneoplastic brain components, which prevents data from being skewed by contaminating cell types as is possible with the other two assays discussed herein.

The Preston Robert Tisch Brain Tumor Center at Duke University Medical Center defines low-level (hence favorable) AGT as <20% cells expressing nuclear AGT staining (4), whereas others set the lower limit at <10% (18) or <5% (29). Defining the therapeutic effect of low level AGT is difficult. Belanich et al. (14), using an immunofluorescence assay, found a striking difference between time to progression and death rate in tumors with <5% AGT versus 5% to 10% AGT, suggesting that an AGT level lower than most defined AGT-low cutoffs could produce a significant level of resistance (14). This may be in part due to the fact that when immunohistochemistry is compared with other methods such as nested *in situ* RT-PCR, there is a 20% discrepancy between the immunohistochemical results and that of *in situ* RT-PCR, suggesting that immunohistochemistry may lack sensitivity and underestimate detectable AGT (18). Consistent with previous studies which estimate that 30% to 40% of tumors lack detectable AGT, we found that 31% of our samples lack significant levels of AGT protein by immunohistochemistry.

In the present study, we observed a strong correlation between AGT protein expression by immunohistochemistry and AGT activity. A positive correlation between biochemical measure of AGT and AGT quantitated by immunoblot and/or RT-PCR has been shown in pediatric brain tumors (40) and brain tumor cells (41), respectively. However, the AGT activity assay requires a large amount of starting material (desired range, 100–300 mg of tumor tissue) and is not only time-consuming but is also not an economically feasible option for many laboratories or in large-scale studies in which tissue procurement is limited. A major advantage of the immunohistochemical assay is that because scoring is done using a microscope, AGT expression can be evaluated specifically in tumor cells. In contrast, it is impossible to establish the source of the AGT activity because tumor homogenates are used in assaying AGT activity by HPLC, unless the specimen is first confirmed histologically to be composed predominantly of tumor cells. Although the HPLC AGT assay introduced in the late 1990s has been used extensively, many investigators have switched to using techniques which require small amounts of starting material, including RT-PCR for AGT gene expression, and MSP for analysis of promoter hypermethylation of the AGT gene.

Hypermethylation of the AGT promoter was detected in 61% of samples, consistent with other recent investigations (22, 27–29, 42). Although many published reports advocate the AGT promoter methylation assay by MSP as an indicator of treatment outcome and overall survival, reported rates of AGT promoter methylation (40–76%) vary widely and are often brighter than other series of tumors immunohistochemically stained for the protein (30–40%). These data would suggest, as we show in this report, a weak albeit statistically significant correlation between the detection of promoter methylation and AGT protein expression in the tumor Although linear regression showed an inverse relationship between immunohistochemistry/AGT activity and MSP (Fig. 1), cross-tabulation studies (Fisher's/McNemar's) indicate a highly significant disagreement between the proportion of patients graded as positive prognosis by immunohistochemistry (<20% staining) and those graded as positive prognosis by MSP (using the AGT/β-actin ratio ≥12), suggesting that one index cannot be used as a predictive tool for another. Most striking was the variable percentage of AGT-negative stained cells in tumors with no evidence of methylation and the high percentage of AGT-positive stained cells in methylation-positive tumors. These findings may indicate the epigenetic and clonal heterogeneity of cells within the glioma tumor. The detection of AGT methylation in a subset of the cells within a tumor will yield a positive methylation assay result, although the remaining cells are capable of producing AGT, resulting in a positive result for immunohistochemistry. Thus, discrepant results may be expected. The degree of gene methylation may play an important role in the clinical utility of the MSP assay (to be assessed in an ongoing prospective study) to predict tumor sensitivity to alkylating drugs, although a tight correlation with protein levels and/or enzyme activity may not always be observed. Interestingly, such discordance was seen in the two tumors that had the highest degree of promoter methylation (as seen in Table 2). A tumor with a methylation ratio of 814 (patient no. 1) had 0% cell staining by immunohistochemistry but had 57 fmol/mg activity, whereas another (patient no. 13) with a MSP ratio of 584 displayed 42% immunohistochemical cell staining but 0 fmol/mg activity.

A series of studies have emphasized the challenge and possible pitfalls of interpreting the overall significance of methylation data in tumor samples with potentially high diversity (29, 42–44). The reagents and quality of MSP techniques, as well as the assessment of methylation status by reading gel band intensities, vary widely across laboratories and could significantly contribute to the disparate published results of AGT methylation rates and effects in brain cancer. Rood et al. (42) found methylation in

the promoters from 28 of 37 medulloblastomas. However, increasing concentrations of bisulfite-treated DNA templates were needed in order to amplify the methylated component in 14 of 28 methylated samples, suggesting that a very small proportion of AGT genes were methylated. Blan et al. (27) found that in 68% of gliomas expressing methylation-positive status, 97% also displayed an unmethylated component. These data suggest that a large percentage of tumors are heterogeneous for the expression of AGT.

One explanation for the inconsistencies observed between methylation and expression may be due to monoallelic AGT expression, as suggested by Nakasu et al. (12) and Rood et al. (42) as well as cellular heterogeneity. Although heterogeneity and monoallelic expression are plausible explanations for the discrepancy observed between the proportion of cells showing methylation and those expressing protein or mRNA, the role of additional factors in the regulation of AGT expression remains controversial and largely unexplained (45). The AGT gene spans nearly 300 kb on chromosomal band 10q26. Loss of heterozygosity has been shown in ~70% of glioblastoma multiforme tumors (46–50). In addition to the AGT gene, the loss of heterozygosity region on chromosome 10 includes multiple loci whose deletion or amplification have been associated with the progression of glioma, including candidate tumor suppressor sites, phosphatase and tensin homologue and deleted in malignant brain tumors 1. Again, deletion of one allele may alter the relationship between cells viewed as positive by immunohistochemistry and the level of promoter methylation or activity associated with each cell. Furthermore, the AGT methylation may be associated with the likelihood of methylation of other important genes which convey tumor sensitivity to alkylating drugs, suggesting that AGT methylation may serve as a surrogate for multiple gene silencing activities within the tumor, which may explain the differences in the clinical outcome often observed based on AGT methylation status. In addition, mutagenesis studies have shown that mutation of independent amino acid residues (R128A, Y114E, Y114A, C145A, E172Q, etc.) abolishes or significantly reduces the repair activity of AGT (51, 52). Although the prevalence of these mutations has not been documented in clinical samples, these studies do show a level at which protein expression and unobserved methylation could be mismatched with activity.

Temozolomide is the community standard of care for malignant glioma. Furthermore, the recent publication by Hegi et al. (23) has led some clinicians to seek MSP quantitation of AGT promoter methylation as a means to determine if temozolomide should be used, presumably avoiding this agent if promoter methylation is not seen. This is premature for two reasons. First, as suggested by Rood et al. (42), a robust and reproducible MSP assay needs further clinical outcome validation and, equally important, rigorous standardization, prior to use as a decision-making variable. Second, as our data now show, and as previous data from Rood et al. has suggested (42), tumors which are MSP-negative will include a large fraction of AGT-negative (by immunohistochemistry), and hence, putatively temozolomide-sensitive cells. Thus, the preferred recommendation for patients with MSP-negative tumors might well be the inclusion of additional agents that are not susceptible to AGT activity to target AGT-positive cells, whereas continuing to include temozolomide to treat the AGT-negative cells. Of course, one could advance the argument that all patients with malignant glioma, and indeed, all malignancies deserve more than monoagent treatment, and that promising multidrug regimens with different modes of antitumor activity need to be explored.

We have shown for the first time a side-by-side comparison of these three independent quantitations for AGT in clinical glioma samples which included the real-time assessment of AGT promoter methylation as opposed to the traditionally used gel-based assay. The data show (*a*) a highly significant correlation between AGT protein expression assessed by immunohistochemistry and AGT activity assessed by HPLC, (*b*) a marginal statistically significant correlation between immunohistochemistry and MSP, and (*c*) no significant correlation between AGT protein activity assessed by HPLC versus MSP. This present study involved a small number of samples and did not include clinical outcome data, thus the results cannot be used to guide glioma therapy decisions. Prediction of treatment outcome in glioma patients has been reported for both immunohistochemical and MSP testing and it is important to recognize that AGT-negative (by immunohistochemistry) cells may reside in tumors with no evidence of AGT methylation (MSP negative). The variable results reported in the literature of AGT gene methylation analysis stresses the importance for establishment of a standardized MSP assay with proven clinical outcome predictability. Oncomethylome Sciences is currently testing its real-time MSP assay in a large, international prospective trial in which glioma patients are stratified by AGT methylation status to achieve such validation. The Brain Cancer program at Duke uses the immunohistochemical assay done by its pathology department as it has benn associated with clinical outcome differences (4). Nevertheles, further work in this area is urgently needed to assess the utility of different assays for AGT for optimizing patient treatment including temozolomide therapy.

Acknowledgments

We thank Diane Satterfield and Melissa Ehinger at the Duke Brain Tumor Tissue Bank for their assistance in procuring tissue; and Dr. Stephen Keir for his assistance in this study.

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Mol Cancer Ther 2006;5:2531-2539.