Molecular Features of Adult Glioma Associated with Patient Race/Ethnicity, Age, and a Polymorphism in O^6-Methylguanine-DNA-Methyltransferase

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Abstract

Background: **Risk factors for adult glioma in the San Francisco Bay Area include well-known demographic features such as age and race/ethnicity, and our previous studies indicated that these characteristics are associated with the *TP53* mutation status of patients' tumors. We enlarged our study to assess the relationships of risk factors with *TP53* as well as epidermal growth factor receptor (*EGFR*) and murine double minute-2 (*MDM2*) gene amplification and expression and the germ line Leu84Phe polymorphism in the DNA repair protein O^6-methylguanine-DNA-methyltransferase (MGMT). MGMT expression may depend on the *TP53* status of cells.**
Methods: **Molecular analyses were carried out on 556 incident astrocytic tumors. *MGMT* genotype data were collected on germ line DNA from 260 of these cases.**
Results: **The tumor data confirm the inverse relationships between *TP53* mutation and *MDM2* ($P = 0.04$) or *EGFR* ($P = 0.004$) amplification and that patients whose tumors contain *TP53* mutations are younger than those without ($P < 0.001$). Although there was little difference in age of patient by *EGFR* amplification or expression among glioblastoma multiforme cases, *EGFR* gene amplification was associated with much older age of onset of anaplastic astrocytoma; for example, *EGFR*-amplified anaplastic astrocytoma cases were on average 63 years old compared with 48 years for nonamplified cases ($P = 0.005$). An increased prevalence of *TP53* mutation positive glioblastoma multiforme was noted among nonwhites (African American and Asian) compared with whites (Latino and non-Latino; $P = 0.004$). Carriers of the *MGMT* variant 84Phe allele were significantly less likely to have tumors with TP53 overexpression (odds ratio, 0.30; 95% confidence interval, 0.13-0.71) and somewhat less likely to have tumors with any *TP53* mutation (odds ratio, 0.47; 95% confidence interval, 0.13-1.69) after adjusting for age, gender, and ethnicity. Interestingly, *EGFR* gene amplification and EGFR protein overexpression were also inversely associated with the *MGMT* 84Phe allele.**
Conclusions: **Our results are consistent with ethnic variation in glioma pathogenesis. The data on MGMT show that an inherited factor involving the repair of methylation and other alkylation damage, specifically to the O^6 position of guanine, may be associated with the development of tumors that proceed in their development without *TP53* mutations or accumulation of TP53 protein and possibly also those that do not involve amplification of the *EGFR* locus.** (Cancer Epidemiol Biomarkers Prev 2005;14(7):1774–83)

Introduction

Malignant gliomas in adults are highly heterogeneous tumors of which etiology is unknown for the vast majority of cases. Molecular characterization of gliomas may help to identify causal factors by establishing more homogeneous categories of disease. In a previous study of 164 adults with glioma, we reported that several hallmark risk factors for primary brain tumors were associated with the *TP53* status of patients' tumors. *TP53* mutations in tumors were more common among nonwhites versus whites and women (1). Also, patients with such tumor mutations were on average 6 years younger at glioma diagnosis than those whose tumors did not have *TP53* mutations (1). This finding is consistent with other observations and a well-established model of gliomagenesis that posits both ''primary'' and ''progressive'' subtypes of glioblastoma multiforme (2-7). Briefly, the progressive form of glioblastoma multiformes developing from low-grade astrocytoma or anaplastic astrocytoma generally occurs at earlier ages and has a higher prevalence of *TP53* mutations than *de novo* glioblastoma multiformes.

The predominate type of *TP53* mutation in gliomas is base pair transition (e.g., G>A and C>T; ref. 8). The causes of the transition mutations in brain tumors are unknown but it has been noted that DNA alkylation at the O^6 position of guanine commonly produces these alterations (9, 10) and that alkylating agents are known neurocarcinogens (11). Furthermore, somatic inactivation of the DNA repair protein O^6-methylguanine-DNA-methyltransferase (MGMT), which specifically repairs alkylation damage at O^6-guanine, is associated with *TP53* G>A transitions in human cancers (12, 13). Several germ line polymorphisms have been identified in MGMT (14-17) including Leu84Phe, which has been shown to affect repair activity. Other common heritable *MGMT* alterations occur at codon 53 in exon 3 and codons 143, 178, and 197 in exon 5. Limited studies of phenotypes associated with these single-nucleotide polymorphisms have not revealed marked alterations in the repair function of MGMT variants expressed *in vitro* (18, 19). However, the codon 143 variant was associated with a 2-fold increased risk for lung cancer in one case-control study (20).

Received 2/3/05; revised 4/8/05; accepted 4/25/05.

Grant support: NIH grants R01CA52689, P50CA097257, ES06717, and ES04705.

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Among melanoma patients, increased expression of the MGMT protein was associated with the *MGMT* 84Phe variant (19). Interestingly, in a Japanese study, *MGMT* 84Phe carriers were significantly overrepresented among primary *de novo* glioblastoma multiforme cases compared with controls (21).

Although great attention has been given to *TP53* mutations in glioma, by far the most common form of the disease (i.e., glioblastoma multiforme) arises in older individuals as a primary malignancy and these tumors more often contain epidermal growth factor receptor (*EGFR*) amplification than *TP53* mutations (22). Many of these TP53 wild-type tumors with EGFR overexpression show abnormal TP53 protein accumulation (23), which also indicates a disruption in the function of TP53. The mechanisms giving rise to this aberrant TP53 accumulation in the absence of mutation are obscure (24, 25). One possible mechanism is through the amplification of the murine double minute-2 (*MDM2*) gene (3, 26, 27). Another possibility involves *PTEN* loss through deletion or methylation that would activate AKT, leading to increased expression of MDM2 and concomitant decreased function of TP53 (28-30). In the present study, we investigated interrelationships between *TP53* mutation and protein expression and the amplification and expression of MDM2 and EGFR and their associations with age and race/ethnicity. Because of the importance of MGMT in repair and *TP53* mutations, we also examined associations of patients' tumor markers with constitutive genotypes for *MGMT* Leu84Phe (Leu/Leu, Leu/Phe, and Phe/Phe). We chose to focus on this candidate single-nucleotide polymorphism both because it is the only polymorphism in *MGMT* previously associated with glioma risk (21) and information from a study of expression (19) allowed us to formulate the hypothesis that if 84Phe increases MGMT expression, then 84Phe carriers may be less likely to have tumors with *TP53* mutation.

Materials and Methods

Subjects. Details of case-control ascertainment for these two series of subjects have been presented in detail elsewhere (31-33). We ascertained all adults newly diagnosed with glioma (International Classification of Diseases for Oncology, morphology codes 9380-9481) in six San Francisco Bay Area counties (Alameda, Contra Costa, Marin, San Mateo, San Francisco, and Santa Clara) from August 1991 to April 1994 (series 1) and from May 1997 to August 1999 (series 2). Cases were ascertained within a median of 7 weeks of diagnosis using the Northern California Cancer Center rapid case ascertainment system. Age-, gender-, ethnicity-, and frequency-matched controls were obtained through random digit dialing (described in detail elsewhere; refs. 31, 34). We began collecting blood specimens from willing subjects partway through the first series and asked all participants in series 2 to donate either a blood and/or buccal specimen. Table 1 shows the number of subjects for the current study. All subjects signed study consent forms and the study was approved by the Committee on Human Research of University of California San Francisco.

Neuropathology Review. Pathology specimens were obtained from diagnosing hospitals and all tumors were reviewed by the study neuropathologist; Richard Davis reviewed cases diagnosed between 1991 and 1994 and Kenneth Aldape reviewed cases diagnosed between 1997 and 1999. Tumors were classified according to the WHO criteria described by Kleihues et al. (35, 36). Glioblastoma multiforme corresponds to WHO grade 4, anaplastic astrocytoma to WHO grade 3, and astrocytoma to WHO grade 2. The corresponding International Classification of Diseases for Oncology (2nd edition) codes are 9440 and 9401 for glioblastoma multiforme and anaplastic astrocytoma. For astrocytoma, there is not an exact correspondence between the WHO criteria grade 2 and the International Classification of Diseases for Oncology (2nd edition) code 9400.

Paraffin-Embedded Tissue–Derived DNA Extraction. For DNA extraction, Dr. Aldape (the neuropathologist of the study) mounted one section of each tumor block for routine H&E staining to find areas with maximal appearance of tumor tissue. We gross dissected tumor section with disposable scalpels to ensure all sections used for DNA extractions contained more than 80% tumor tissue. Adjacent four 50-μm microtome sections of paraffin blocks were made, and placed in a 1.5 mL Eppendorf tube. These were deparaffinized after heating in a 65°C water bath for 10 minutes by serial washes with xylene and absolute alcohol. After the final alcohol wash, the tube was centrifuged at 10,000 × g for 5 minutes and the pellet speed dried. The pellet was resuspended in 50 μL of buffer consisting of 500 mmol/L KCl, 100 mmol/L Tris-HCl, 1.0% Triton X-100 with 4.5% NP40, 4.5% Tween 20, and 5 μL of 10 mg/mL proteinase K. Samples were incubated for 1 hour at 55°C, then for 10 minutes at 95°C to inactivate the proteinase. The insoluble material was pelleted by centrifugation, supernatant DNA content was determined by fluorometer, and PCR was done on aliquots of 20 to 50 ng of DNA. Disposable microtome blades were changed between each block to eliminate DNA cross-contamination.

TP53, EGFR, and MDM2 Immunohistochemical Staining. Staining was done as previously described (37). Five-micron sections were deparaffinized in histologic grade xylene for 10 minutes and rehydrated through sequential 95% to 70% ethanol and placed in PBS. Microwave antigen retrieval was done by placing the slides in 50 mmol/L citrate buffer (pH 6.0) and microwaving for 12 minutes, and then given two to three 5-minute washes in PBS. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide in PBS/0.05% Tween 20 or 3% hydrogen peroxide in methanol for 10 to 20 minutes. Sections were then washed two to three times in PBS and blocked for 20 minutes in the appropriate serum from the same species as the secondary antibody diluted to 10% in PBS. The anti-TP53 (DO-7, Dako, Carpinteria, CA) mouse monoclonal antibody, 1:150 diluted in PBS/10% serum, was applied to the sections in a humid chamber for 2 hours at room temperature or overnight at 4°C. After washing two to three times in PBS, the secondary antibody was applied per directions in a kit from Vector Labs (Burlingame, CA). Briefly, biotinylated anti-mouse was diluted in 10% normal horse serum/PBS (1:200) and sections incubated at room temperature for 30 minutes. Detection of the antibody was done with 3,3′-diaminobenzidine for 1 to 5 minutes. For EGFR and MDM2, primary antibodies used were anti-EGFR (Ab-1, clone 528) and anti-MDM2 (Ab-1), both from Oncogene Research Products (San Diego, CA). Sections were then counterstained with light hematoxylin and mounted. Scoring for TP53 or MDM2 was for nuclear staining on a four-point scale from 0 to 3. A score of 0 indicated no staining, 1 indicated less than 5% of nuclei with positive staining, 2 indicated 5% to 30% positive nuclei staining, and 3 indicated greater than 30% positive nuclei staining. Scoring for EGFR was for membrane/cytoplasmic staining on a three-point scale, where 0, no staining; 1, weak/focal staining; and 2, strong/diffuse staining.

***TP53* Mutation Analyses.** PCR-single strand conformation polymorphism assay and DNA sequencing were used to determine the frequency of *TP53* mutations. Briefly, oligonucleotide primers for PCR amplification of fragments of exons 5 to 8 were synthesized by Operon Technology, Inc. (Alameda, CA). Sequences for primers used were 5′-gttcacttgtgccctga-3′ and 5′-agccctgtcgtctct-3′ for exon 5, 5′-ctctgattcctcactg-3′ and 5′-ccagagaccccagttgcaaacc-3′ for exon 6, 5′-tgcttgccacaggtct-3′ and 5′-acagcaggccagtgt-3′ for exon 7, and 5′-aggacctgatttccttac-3′ and 5′-tctgaggcataactgc-3′ for exon 8. PCR products were generated in a 30 μL reaction mixture including 50 ng DNA, 20 μmol/L deoxynucleotide triphosphate, 10 mmol/L

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Table 1. Description of people with astrocytic tumors from the San Francisco Bay Area Adult Glioma Study, 1991-2000

	All astrocytic cases, *n* (%)	Astrocytic cases with tumor results, *n* (%)
	N = 667	*N* = 556
Gender		
Male	371 (56)	322 (58)
Female	296 (44)	234 (42)
Histology		
Glioblastoma multiforme	519 (78)	441 (79)
Anaplastic Astrocytoma	105 (16)	86 (15)
Astrocytoma	43 (6)	29 (5)
Race		
White	549 (82)	463 (83)
Latino	38 (6)	35 (6)
Nonwhite	80 (12)	58 (10)
Age		
Median	59	59
Mean	58.8	59.2
SE	0.58	0.63
MGMT Leu84Phe genotype	*N* = 305	*N* = 260
CC (wt/wt)	236 (77)	200 (77)
CT (wt/var)	63 (21)	55 (21)
TT (var/var)	6 (2)	5 (2)

Tris-HCl (pH 9.0), 1.5 mmol/L $MgCl_2$, 0.1% Triton X-100, 10 pmol of each primer, 1 unit Taq (Perkin-Elmer Cetus, Norwalk, CT), and 0.2 μCi [^{33}P]dCTP (DuPont New England Nuclear, Boston, MA). DNA with known *TP53* mutation was included as positive control. The PCR reaction was carried out using 35 cycles (94°C for 30 seconds, annealed for 30 seconds at various temperature as indicated in Table 1, and 72°C for 1 minute) on a Perkin-Elmer 9600 thermal cycler. Three microliters of PCR product were mixed with 2 μL of 0.1 N NaOH and then mixed with 5 μL of gel loading buffer solution from United States Biochemical Corp. (Cleveland, OH) and heated at 94°C for 4 minutes. Samples were kept on ice and loaded immediately onto 6% nondenatured polyacrylamide gel supplemented with 10% glycerol. Gels were run at room temperature for 20 hours and exposed for 16 hours for autoradiographic detection of bands. Direct sequencing of PCR fragments for both DNA strands was done on all tumor DNAs with aberrant migration patterns on single strand conformation polymorphism gel to determine the corresponding DNA sequences using dsDNA cycle sequencing system from Life Technologies (Gaithersburg, MD).

***EGFR* and *MDM2* Gene Amplification.** *EGFR* and *MDM2* amplification was measured by a quantitative PCR method (ABI 7900) using the generic DNA binding dye SYBR Green I, which has been shown to be equivalent to TaqMan to assess gene copy number (38). Quality control measures for the real-time SYBER green assay included running triplicate determinations for both the target (*EGFR* or *MDM2*) and control genes (*GAPDH*). The patient DNA was extracted from paraffin-embedded tumor tissue in parallel with normal DNA extracted from paraffin-embedded normal tissue. Cell line DNAs served as positive and negative controls for amplification that were run with each experiment. We used the cell lines A431 (amplified for *EGFR*), HT29 (negative *EGFR* amplification), Rh18 (*MDM2* amplified), and Rh30 (negative *MDM2* amplification). A431 and HT29 were obtained from the American Type Tissue Culture Association. Rh18 and Rh30 were generously provided by Dr. Peter Houghton of St. Jude Children's Research Hospital (Memphis, TN).

Real-time PCR. Primers for real-time PCR were designed by using Primer Expression version 1.5 software (Applied Biosystems, Foster City, CA). The housekeeping gene *GAPDH* was used as an internal control for differences in DNA concentration. For each sample, the gene of interest and *GAPDH* were both amplified in triplicate, and results were analyzed by using Sequence Detector version 1.7 and Dissociation curve version 1.0 software (Applied Biosystems). Relative quantification was done with the standard curve method, and gene amplification levels were normalized by dividing by *GAPDH* levels in each sample. A cutoff of three copies was considered amplified. Haploid copy numbers were compared by ΔΔCT method (38) for the mean cycle threshold (CT) of the reaction triplicates as follows: $2^{-\Delta\Delta CT} = [(1 + E)^{-\Delta CTgene}] / [(1 + E)^{-\Delta CTreference\ gene}]$, where E is the efficiency of the PCR reaction (set at default value 0.95), ΔCTgene is the difference in threshold cycle value between test sample and calibrator sample (BT71) for the gene under investigation (test gene), and ΔCTreference gene is difference in threshold cycle value between test sample and calibrator sample (BT71) for reference gene (*GAPDH*). Primers were *EGFR* forward: 5′CCGCATTAGCTCTTAGACCCA, *EGFR* reverse: 5′GAATGCAACTTCCCAAAATGTGC, *MDM2* forward: 5′TCCCCGTGAAGGAAACTGG, *MDM2* reverse: 5′TTTCGCGCTTGGAGTCG, *GAPDH* forward: 5′CTCCCCACACACATGCACTTA, *GAPDH* reverse: 5′CCTAGTCCCAGGGCTTTGATT.

***MGMT* Genotyping.** *MGMT-L84F* genotype was determined using an allelic discrimination 5′-nuclease assay (TaqMan) on the ABI PRISM 7000 Sequence Detection System (Applied Biosystems) in 96-well format. TaqMan primers and probes were designed using the Primer Oligo Design Software v2.0 (Applied Biosystems). Primers employed for the amplification of *MGMT-L84F* were *MGMT-L84F* forward: CCCGAGGCTATCGAAGAGTTC and *MGMT-L84F* reverse: CCGACCTTGCTGGAAAACG. Probes used for detection of *MGMT-L84F* single-nucleotide polymorphism were vic-ATGGTGAAGAGCCGG-NFQ and fam-ATGGTGAAAAGCCGG-NFQ.

Statistical Analyses. Fisher's exact and χ^2 tests were used to evaluate associations between the presence of tumor markers and subject's age, race, gender, and glioma histopathologic subtype. Logistic analyses were used to estimate the odds ratio (OR) for association of variables and markers in multivariate analyses. Log-linear analyses were conducted to evaluate whether joint proportions of markers differed from expected assuming independence. Statistical analyses were conducted using SAS software for personal computers.

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Results

Tissue Specimens. Tumor tissues sufficient for molecular studies were available for 556 of 667 (83%) subjects with astrocytic tumors and of these, 260 provided blood or buccal specimens for studies of germ line DNA. As shown in Table 1, the distribution of our overall case population was almost identical with respect to gender, histology, race/ethnicity, age, and *MGMT* genotype as 83% of cases with sufficient tumor material for marker evaluation. Although the population was mostly of white race/ethnicity (82%), we did collect extensive data on 35 white Latino and 58 nonwhite cases. Genotype frequencies for *MGMT* Leu84Phe among 526 controls were 80% Leu/Leu, 18% Leu/Phe, and 2% Phe/Phe, very similar to that observed for the astrocytic glioma cases (Table 1).

Relationship of Tumor Markers to One Another and Histology. Table 2 shows that the frequencies of presence or absence of each of the markers varied significantly by histologic grade and, as expected, *TP53* mutations were about 2-fold more common among astrocytoma and anaplastic astrocytoma tumors compared with glioblastoma multiforme. Conversely, *EGFR* amplification and overexpression were most common in glioblastoma multiformes. The most prevalent genetic alteration in glioblastoma multiforme was *EGFR* amplification (36% of tumors) and the most common abnormal protein expression was TP53 (83%). For both anaplastic astrocytoma and astrocytoma, the most common genetic alteration was *TP53* mutation (36% for anaplastic astrocytoma and 29% for astrocytoma) and the most common abnormal protein accumulation was TP53 (73% for anaplastic astrocytoma and 75% for astrocytoma).

Table 3 shows the pairwise relationships [estimated as ORs and 95% confidence intervals (95% CI)] among the tumor markers in glioblastoma multiforme patients. We limited these analyses of associations of markers to glioblastoma multiforme because of the relatively large numbers of subjects with this diagnosis. Also, note that numbers of subjects for different combinations differ because not all markers were available for each subject due to limited tumor specimens or inadequate quality of some specimens for all marker analyses. We observed highly statistically significant associations between *TP53* mutation and TP53 protein overexpression with a trend of increasing strength of association across expression levels. ORs for mutation increased from the medium expression level (5-30% of cells express TP53) to the high level (>30% of cells express TP53). These trends were not significantly affected by the few tumors (n = 3) that contained truncating mutations in *TP53* that were also immunohistochemically negative. Amplifications of the *MDM2* and *EGFR* loci were both inversely associated with *TP53* mutation. EGFR protein overexpression was also inversely related to *TP53* mutation and the most intense immunohistochemical staining for TP53 protein but not for any or moderate TP53 protein overexpression. The amplification of *MDM2* was marginally associated with MDM2 protein accumulation; some MDM2 protein–positive tumors did not show detectable *MDM2* amplification, which itself was relatively uncommon (9.0% overall). In contrast, most of the tumors that overexpressed EGFR protein (n = 42) also had *EGFR* amplification (n = 33); amplified tumors were 25 times more likely to overexpress protein detectable by immunohistochemistry.

Given the relatively large numbers of glioblastoma multiforme subjects available with all tumor markers, patterns of combinations of markers are also of interest (Fig. 1A and B). Of the eight possible combinations of presence or absence of tumor genetic alterations (*TP53* mutation, *EGFR* and *MDM2* amplification), only three combinations individually held 10% or more of tumors (Fig. 1A); specifically, 46% of tumors had no detectable *TP53* mutation nor *MDM2* or *EGFR* amplification, 31% of tumors had *EGFR* amplification only, and 12% of tumors had *TP53* mutation only. There was at least one tumor for other possible combinations, except that no tumors had alterations in all three genes. Of the eight possible combinations of presence or absence of tumor accumulation of TP53, EGFR, or MDM2 proteins, only three combinations individually held 10% or more of tumors (Fig. 1B); 31% of tumors had accumulation of both TP53 and MDM2; 30% of tumors had accumulation of TP53, EGFR, and MDM2; and 13% had accumulation of TP53 only. From 3% to 9% of tumors had each of the other five possible patterns of accumulation. Log-linear models suggest significant two-way deviations from independence: there were significantly fewer tumors containing both *EGFR* amplification and *TP53* mutations (P = 0.004) than expected based on chance. We noted that in those uncommon tumors that did contain both *EGFR* amplification and *TP53* mutation (10 of 368, 2.7%), the spectrum of *TP53* mutation was not different from the more common tumors (43 of 368, 11.7%) that carried only *TP53* mutation and no *EGFR* amplification (data not shown). We also found fewer tumors with both *MDM2* amplification and *TP53* mutation (P = 0.09) than expected. There were significantly more tumors than expected overexpressing both MDM2 and TP53 proteins. Among 345 glioblastoma multiforme tumors in which all six tumor markers were measured, only 2 of 64 possible combinations of presence or absence of the marker contained 10% or more of tumors. Specifically, 68 tumors (20%) had no *TP53* mutation nor *MDM2* or *EGFR* amplification with accumulation of both TP53 and MDM2, but not EGFR, proteins; 64 tumors (19%) had only *EGFR* amplification accompanied by accumulation of

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Table 2. Comparison of prevalence of tumor markers by histologic type, San Francisco Bay Area Adult Glioma Study, 1991-2000

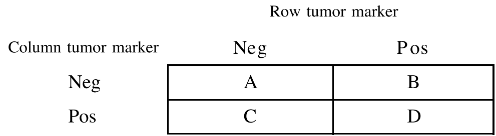
	Glioblastoma	Anaplastic astrocytoma	Astrocytoma	P
	positive/total n (%)	positive/total n (%)	positive/total n (%)	
TP53 mutation*	62/409 (15)	27/76 (36)	7/24 (29)	<0.001
TP53 immunohistochemistry†	365/438 (83)	62/85 (73)	21/28 (75)	0.05
EGFR amplification‡	140/386 (36)	14/68 (21)	2/18 (11)	0.005
EGFR immunohistochemistry§	194/412 (47)	27/82 (33)	3/27 (11)	<0.001
MDM2 amplification‖	38/386 (10)	1/68 (1)	3/20 (15)	0.05
MDM2 immunohistochemistry¶	292/415 (70)	34/81 (42)	5/27 (19)	<0.001

*TP53 mutation versus no mutation.
† TP53 immunohistochemistry, any stain (1, 2, or 3) vs no stain (0).
‡ EGFR amplified (copy number >3.0) vs nonamplified (copy number ≤ 3.0).
§EGFR immunohistochemistry, any stain (1, 2, 3) vs no stain (0).
‖MDM2 amplified (copy number >3.0) vs nonamplified (copy number ≤ 3.0).
¶MDM2 immunohistochemistry, any stain (1, 2, 3) vs no stain (0).

Table 3. Matrix of associations of molecular markers in tumors histologically classified as glioblastoma multiforme; the San Francisco Bay Area Adult Glioma Study, 1991-2000

	TP53 immunohisto-chemistry, all*	TP53 immunohisto-chemistry, med[†]	TP53 immunohisto-chemistry, high[‡]	MDM2 amplification[§]	MDM2 immunohisto-chemistry[‖]	EGFR amplification[¶]	EGFR immunohisto-chemistry[**]
TP53 mutation [††]	**4.7 (1.4-15.3) 0.01 N = 407 (66, 279, 3, 59)**	**7.2 (2.2-23.8) <0.001 N = 288 (66, 165, 3, 54)**	**15.9 (4.7-53.7) <0.001 N = 181 (66, 65, 3, 47)**	**0.16 (0.02-1.2) 0.04 N = 372 (284, 33, 54, 1)**	0.91 (0.49-1.7) 0.75 N = 387 (94, 237, 17, 39)	**0.37 (0.19-0.75) 0.004 N = 375 (193, 126, 45, 11)**	**0.49 (0.27-0.90) 0.02 N = 384 (167, 161, 38, 18)**
TP53 immunohisto-chemistry, all*		‡‡ ‡‡ ‡‡ ‡‡	‡‡ ‡‡ ‡‡ ‡‡	1.0 (0.41-2.6) 1.0 N = 384 (56, 6, 290, 32)	**2.1 (1.2-3.5) 0.01 N = 414 (30, 39, 93, 252)**	0.98 (0.56-1.7) 1.0 N = 384 (40, 23, 205, 116)	0.99 (0.59-1.7) 1.0 N = 411 (37, 33, 181, 160)
TP53 immunohisto-chemistry, med[†]			‡‡ ‡‡ ‡‡ ‡‡	1.0 (0.40-2.7) 1.0 N = 272 (56, 6, 189, 21)	**2.1 (1.2-3.7) 0.01 N = 296 (30, 39, 60, 167)**	0.80 (0.44-1.4) 0.54 N = 272 (40, 23, 143, 66)	0.92 (0.54-1.6) 0.78 N = 294 (37, 33, 123, 101)
TP53 immunohisto-chemistry, high[‡]				0.73 (0.24-2.2) 0.57 N = 172 (56, 6, 102, 8)	**2.1 (1.1-3.9) 0.02 N = 184 (30, 39, 31, 84)**	**0.44 (0.22-0.88) 0.03 N = 172 (40, 23, 87, 22)**	0.60 (0.33-1.1) 0.12 N = 185 (37, 33, 75, 40)
MDM2 amplification[§]					**2.2 (0.91-5.6) 0.09 N = 365 (97, 230, 6, 32)**	0.77 (0.37-1.6) 0.59 N = 379 (218, 125, 25, 11)	0.54 (0.26-1.1) 0.11 N = 362 (169, 157, 24, 12)
MDM2 immunohisto-chemistry[‖]						1.1 (0.70-1.8) 0.63 N = 365 (66, 165, 35, 99)	**1.5 (0.97-2.3) 0.08 N = 407 (71, 48, 143, 145)**
EGFR amplification[¶]							**25.0 (13.5-46.2) <0.001 N = 364 (179, 55, 15, 115)**

NOTE: Associations measured by raw OR and 95% CI (first line of each cell). Fisher's exact two-sided P value on second line of each cell. Values in bold: $P < 0.10$.
* TP53 immunohistochemistry, no stain (0) vs any stain (1, 2, or 3).
† TP53 immunohistochemistry, no stain (0) vs at least medium stain (2 or 3); stain = 1 omitted.
‡ TP53 immunohistochemistry, no stain (0) vs highly stained (3); stain = 1 or 2 omitted.
§ MDM2 nonamplified (copy number ≤ 3.0) vs amplified (copy number > 3.0).
‖ MDM2 immunohistochemistry, no stain (0) vs any stain (1, 2, 3).
¶ EGFR nonamplified (copy number ≤ 3.0) vs amplified (copy number > 3.0).
**EGFR immunohistochemistry, no stain (0) vs any stain (1, 2, 3).
†† TP53 no mutation versus any mutation.
‡‡ No statistics calculated for these association because the variables are subsets of one another. See coding table legend.



Column tumor marker	Row tumor marker: Neg	Row tumor marker: Pos
Neg	A	B
Pos	C	D

In this format: (A, B, C, D) for fourth line of each cell

TP53, EGFR, and MDM2 proteins. There were other 34 possible combinations of the six markers represented in at least one tumor, with numbers (and percentages) of tumors in each of the 34 combinations ranging from 1 (0.29%) to 27 (7.8%; data not shown).

Tumor Markers and Age at Diagnosis. Table 4 shows associations of age of patient with TP53 and other markers. Patients whose tumors had *TP53* mutation were on average 8 years younger at diagnosis among all astrocytic cases. The lower grade anaplastic astrocytomas with *TP53* mutations were 10 years younger on average compared with cases that did not have mutations and the glioblastoma multiforme cases with *TP53* mutations were 4 years younger on average. In contrast, the ages of TP53 immunohistochemically positive and negative cases were not markedly different from one another for glioblastoma multiforme but were so for anaplastic astrocytomas. Anaplastic astrocytoma patients with TP53 immunohistochemically positive tumors were about 8 years younger than those with negative immunohistochemistry. Average ages of glioblastoma multiforme cases whose tumors had *EGFR* amplification or overexpression were not statistically significantly different from those without these features. However, the mean age of anaplastic astrocytoma cases with *EGFR* amplification or immunohistochemically overexpressing tumors was 15 and 5 years older, respectively, than patients whose tumors did not have *EGFR* amplification or accumulation. Also noteworthy is that the average age for patients with anaplastic astrocytoma with *EGFR* amplification (63 years old) was very similar to

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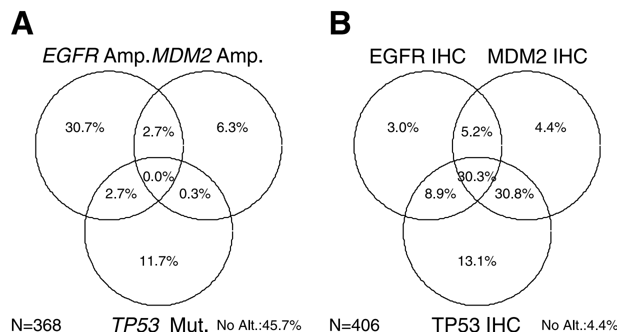

Figure 1. A. Percentages of tumors with combinations of *TP53* mutation and *MDM2* and *EGFR* amplification. **B.** Percentages of tumors with combinations of TP53, MDM2, and EGFR protein as measured by immunohistochemistry. Total within Venn diagram plus those with no alterations equals 100%. *N,* total number of glioblastoma tumors.

the average age at diagnosis for glioblastoma multiforme patients, regardless of tumor *EGFR* status (62 years old). MDM2 overexpression was associated with somewhat greater age among patients.

Tumor Markers and Race/Ethnicity. We also examined molecular characteristic of tumors by race/ethnicity. Only tumor *TP53* mutation status and EGFR protein accumulation significantly varied by race/ethnicity. In particular, African Americans, Asians, and those of other ethnicity had a higher proportion of tumors with *TP53* mutation (17 of 50, 34%) than Latino and non-Latino whites (79 of 459, 17%): age-adjusted OR, 2.6 (95% CI, 1.3-4.9); P = 0.005. In contrast, a lower percentage of African Americans, Asians, and those of other ethnicity had tumors with EGFR expression (15 of 52, 29%) than Latino and non-Latino whites (209 of 469, 44%): age-adjusted OR, 0.51 (95% CI, 0.27-0.96); P = 0.04. Interestingly, tumor *MDM2* amplification was nearly twice as common among Asians (4 of 19 tumors, 21%) compared with other ethnic groups in which 6% to 11% of tumors had *MDM2* amplification, but the difference in proportions was not significant.

Tumor Markers and Constitutive *MGMT* Genotypes. As noted above, the overall *MGMT* Leu84Phe genotype frequencies were similar for the 260 cases with tumor markers and all cases (23% were *MGMT* 84Phe carriers) and controls (20% were *MGMT* 84Phe carriers). Among glioblastoma multiforme cases, 23%, 11%, and 5% of patients whose tumors had no *TP53* mutation, any *TP53* mutation, or only *TP53* G:C transition mutations were *MGMT* 84Phe carriers (Table 5). The results are based on small numbers of observations and cannot be definitive but are consistent with the hypothesis that the *MGMT* 84Phe allele is less common among glioma patients whose tumors contain G>A or C>T transition type base substitutions.

TP53 overexpression was consistent with the mutation data and was highly significantly associated with the *MGMT* 84Phe variant genotypes (P = 0.006, Table 5). Restricting the analysis to whites with glioblastoma, the percentages of subjects carrying *MGMT* 84Phe were 39% (12 of 31), 20% (11 of 54), 7% (3 of 42), and 13% (5 of 39) for those whose tumors had 0%, >0% to 5%, >5% to 30%, and >30% of cells staining for TP53 (χ^2 test comparing four percentages, P = 0.005).

We also examined *MGMT* 84Phe prevalence according to both *TP53* mutation and expression. Only 1 of 18 (5%) people with *TP53* mutant tumors with intense staining had a 84Phe allele compared with 12 of 29 (41%) people whose tumors contained neither *TP53* mutation nor staining; this represents a 7.8-fold difference in carrier prevalence. However, only 8% (3 of 36) of people had tumors with moderate TP53 expression and no *TP53* mutations were *MGMT* 84Phe carriers. These results could be interpreted to indicate that the *MGMT* 84Phe allele is overrepresented among cases with no TP53 staining and less common among tumors with moderate or intense TP53 immunohistochemical staining.

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Table 4. Associations of tumor molecular markers with age by tumor histology, the San Francisco Bay Area Adult Glioma Study, 1991-2000

	All astrocytic cases					Glioblastoma multiforme					Anaplastic astrocytoma				
	n	Mean age	SE	*P*	OR*	*n*	Mean age	SE	*P*	OR*	*n*	Mean age	SE	*P*	OR*
TP53 no mutation	413	61	0.7			347	62	0.7			49	54	2.0		
TP53 with any mutation	96	53	1.7	**<0.001**	**0.96 (0.95-0.98)**	62	58	2.0	**0.02**	**0.98 (0.96-1.00)**	27	44	3.1	**0.01**	**0.95 (0.91-0.99)**
TP53 immunohistochemistry, neg (0)	103	59	1.4			73	61	1.6			23	57	2.8		
TP53 immunohistochemistry, pos (1, 2, 3)	448	59	0.7	0.78	1.00 (0.98-1.01)	365	62	0.7	0.93	1.00 (0.98-1.02)	62	49	2.0	**0.04**	**0.97 (0.94-1.00)**
EGFR non amplified (copy number ≤ 3.0)	316	59	0.9			246	63	0.9			54	48	2.0		
EGFR amplified (copy number >3.0)	156	60	0.9	0.50	1.00 (0.99-1.02)	140	60	1.0	0.06	0.99 (0.97-1.00)	14	63	3.3	**0.005**	**1.08 (1.02-1.14)**
EGFR immunohistochemistry, neg (0)	297	59	0.9			218	62	0.9			55	50	2.2		
EGFR immunohistochemistry, pos (1, 2, 3)	224	60	0.9	0.26	1.01 (1.00-1.02)	194	61	0.9	0.25	0.99 (0.98-1.01)	27	55	2.6	0.14	1.02 (0.99-1.06)
MDM2 non amplified (copy number ≤ 3.0)	432	59	0.7			348	61	0.7			67	51	1.9		
MDM2 amplified (copy number >3.0)	42	62	2.2	0.17	1.02 (0.99-1.04)	38	64	2.0	0.26	1.02 (0.99-1.04)	1	69	–	0.39	1.05 (0.94-1.2)
MDM2 immunohistochemistry, neg (0)	192	57	1.1			123	62	1.2			47	51	2.3		
MDM2 immunohistochemistry, pos (1, 2, 3)	331	60	0.8	**0.04**	**1.01 (1.00-1.03)**	292	62	0.8	0.58	1.00 (0.98-1.01)	34	52	2.6	0.80	1.00 (0.98-1.03)

NOTE: Boldface values: P < 0.05.

*OR (95% CI) per year of age for people with versus without the marker. OR adjusted for age, race/ethnicity (white vs nonwhite), and gender.

Table 5. Inverse association of constitutive MGMT Leu84Phe polymorphism with tumor TP53 mutation and immunohistochemistry among people with glioblastoma multiforme of all races/ethnicity, the San Francisco Bay Area Adult Glioma Study, 1991-2000

Molecular outcome	MGMT Leu84Phe		OR (95% CI)	*P*
	CC	CT or TT		
TP53 no mutation	120	35	1.0	
TP53 with any mutation	24	3	0.47 (0.13-1.69)	0.25
TP53 no mutation	120	35	1.0	
TP53 transition mutation, G/A or C/T only	18	1	0.18 (0.02-1.46)	0.11
TP53 immunohistochemistry, neg (0)	22	13	**1.0**	
TP53 immunohistochemistry, pos (1, 2, 3)	134	25	**0.30 (0.13-0.71)**	**0.006**

NOTE: Boldface values: $P \leq 0.10$. ORs adjusted for series, gender, age, and race/ethnicity (white vs nonwhite).

Finally, we assessed germ line *MGMT* genotype in relation to the *EGFR* status of tumors. Adjusting for series, gender, age, and race, the *MGMT* 84Phe allele was somewhat less common among cases whose tumors contained *EGFR* amplification (OR, 0.50; 95% CI, 0.22-1.13) or overexpressed the EGFR protein (OR, 0.61; 95% CI, 0.29-1.28), compared with cases whose tumors did not have alterations in EGFR. No significant associations were noted for *MGMT* genotype with any other marker.

Discussion

The histologic, age, and gender distributions of these population-based cases are similar to those expected based on U.S. registry data (39). Because brain tumors are relatively rare, even with many years of accrual, glioblastoma multiforme was the only histologic subgroup with sufficient numbers of subjects for meaningful comparisons of tumor markers and constitutive polymorphisms. Another limitation was that even with rapid case ascertainment, we obtained blood or buccal specimens from 96 of 305 (31%) subjects with molecular tumor markers for series 1 and 164 of 251 (65%) for series 2, owing to both the poor survival for most glioma patients and to our obtaining funding for blood collection only partway through the first series. Nevertheless, this study is among the largest astrocytic glioma series assembled to date with uniform neuropathology review to explore etiologic factors associated with molecular subgroups of tumors (40).

We chose the *TP53* mutational status of tumors as the initial molecular feature to subclassify gliomas in our Bay Area glioma population. The current data confirm our previous observation (1) that *TP53* mutations in astrocytic gliomas are more frequent among individuals of nonwhite race/ancestry and among those with earlier age of onset.

Because a mutually exclusive relationship may exist between the disruption of the TP53 pathway and the overexpression of the *EGFR* gene within gliomas, we predicted that *EGFR* amplification or EGFR overexpression would mark a group of glioma patients with race/ethnicity and age characteristics different from those cases whose tumors contain *TP53* alterations. We first explored the relationships of *TP53* mutation and expression with *EGFR* amplification and expression. The presence of *TP53* mutation or high TP53 protein accumulation was strongly inversely associated with *EGFR* amplification or expression (as expected). In contrast, low to moderate TP53 accumulation was only slightly inversely associated with *EGFR* amplification or overexpression. In other words, TP53 expression in up to 30% of nuclei co-occurred with *EGFR* amplification and overexpression. This suggests that some accumulation of TP53 is not mutually exclusive with *EGFR* amplification or overexpression but that *TP53* mutation and high TP53 accumulation (usually associated with mutation) are highly inversely correlated with EGFR alterations. We also report that the mutation spectrum in the uncommon tumors that contained both *TP53* mutation and *EGFR* amplification is typical of *TP53* mutations found overall.

Age at diagnosis was strongly associated with *EGFR* amplification among patients with anaplastic astrocytomas; those with versus those without *EGFR* amplification were 63 years versus 48 years old on average at diagnosis. The marked differences in age of onset for these anaplastic astrocytomas could be a clue to a common etiologic factor operating in some patients with this histologic subtype. Alternatively, it is possible that anaplastic astrocytomas with *EGFR* amplification in these older patients are actually unrecognized glioblastoma multiforme tumors. This may occur when there is inadequate biopsy material for detailed pathology study and a lack of some diagnostic criteria for glioblastoma multiforme; thus, a default diagnosis of anaplastic astrocytoma may be made. It also is possible that some anaplastic astrocytomas may only have the histologic features of grade 3 tumors but the molecular features of grade 4 tumors. Our observation is supported by a previous study indicating that patients with anaplastic astrocytoma tumors showing *EGFR* amplification have survival times comparable with glioblastoma multiforme patients (41). Further study of our finding is warranted as it suggests that *EGFR* amplification could be a rapid means of evaluating some incompletely sampled anaplastic astrocytoma tumors in older patients and of improving diagnostic accuracy. If older age onset anaplastic astrocytomas with *EGFR* amplification are actually misclassified glioblastoma, it might in part explain why there is little difference in survival among older age onset anaplastic astrocytoma versus glioblastoma multiforme compared with substantial grade differences in survival among patients of younger age of disease onset (for example, relative 2-year survival rates for glioblastoma multiforme versus anaplastic astrocytoma are 8% versus 32% for those of ages 45-64 at diagnosis, but 2% versus 6% for those of ages 65 and over; ref. 42).

Accumulation of the EGFR protein identified by immunohistochemistry was also associated with an older age at disease diagnosis; 55 and 50 years for EGFR immunohistochemically positive and negative tumors, respectively. The EGFR protein marker, as in the case of immunohistochemical detection of the TP53 protein, was less strongly related to differences in age at diagnosis compared with the marker of molecular alteration at genetic level (i.e., *EGFR* amplification or *TP53* mutation). Despite a very strong correlation of *EGFR* amplification with protein accumulation, it seems that the two end points are sensitive to different aspects of the age-dependent risk for glioma. Moreover, the use of *TP53* mutation and *EGFR* amplification to subdivide glioma, although related to patient age, is complicated by the fact that both alterations may occur in small cellular compartments within the same lesion (43). This fact further complicates efforts to create mutually exclusive categories of glioma.

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Conventional molecular methods applied to archived paraffin-embedded tumors, as used here, cannot detect these subtle effects. It is also noteworthy that independent of these considerations, the single largest category of glioblastoma multiformes (46% of tumors) contained neither *TP53* mutation nor *EGFR* or *MDM2* amplification. Thus, although at least two molecular pathways in gliomagenesis have been recognized for some time, other important subtypes are likely to exist (43a). Possibilities include deregulation of the TP53 or related pathways through loss of other genes such as *PTEN* and *p14ARF* and aberrant methylation of regulatory genes (increased methylation of tumor suppressing genes or decreased methylation of oncogenes; refs. 44-49).

The current study further examined the potential role of race/ethnicity in the molecular features of adult glioma. Our results indicated a higher prevalence of *TP53* mutations among 50 nonwhite cases compared with 459 white cases that included 33 Latino white individuals. Interestingly, the *TP53* mutation rate among tumors from Latinos seemed similar to that of white non-Latinos. We also found that EGFR overexpression was less common and *MDM2* amplification was somewhat more common among nonwhites. These findings further support the idea that the proportions of glioma cases with different molecular pathways vary by race/ethnicity. These trends are based on small numbers and must be viewed cautiously but are consistent with a shift in molecular subtype of glioma in white compared with nonwhites on the occurrence of *TP53* mutation, *MDM2* amplification, and lower prevalence of EGFR overexpression. The possibility that race/ethnicity may be associated with cancer phenotype at the molecular level could open new avenues for exploring glioma etiology (50).

The occurrence and molecular signature of *TP53* mutations have been proposed as a biomarker of potential carcinogen exposures in many cancers. In adult glioma, however, the mutational spectrum has been described as being typical of an ''endogenous'' pattern of mutation that shows no specific carcinogen-related alterations or hotspots. Here we observed the expected distribution of *TP53* alterations (i.e., a predominance of base substitution type G>A and T>C transitions). These account for about 40% of *TP53* mutations in this disease. However, this pattern of mutation is also consistent with exposure to well-known neurocarcinogens that can induce these alterations. For example, nitrosamides in animals are capable of inducing central nervous system cancers (9, 51-53), and the ability of these compounds to cross the blood-brain barrier and form alkylation products in DNA, including O^6-methylguanine, is considered crucial. An early event in the induction of brain tumors by nitrosourea in mice is the loss of TP53 function (54). Further strengthening the potential link between O^6-methylguanine lesions and the mutational spectrum of gliomas are recent studies showing that acquired MGMT inactivation (via aberrant methylation) within tumors is associated with increased frequencies of transition type mutations (e.g., G:C>A:T) in the *TP53* gene (12, 13). Consistent with the hypothesis that the *MGMT* 84Phe allele is linked with higher MGMT repair activity (19), we found this allele to be underrepresented among glioblastoma multiforme cases whose tumors contained *TP53* mutations, although the OR for those with versus those without transition mutations was only of borderline statistical significance (i.e., $P = 0.11$). It is important to note that MGMT repair is likely to affect the induction of similar base substitutions within several or many genes involved in carcinogenesis; thus, it may not be surprising that analysis of *TP53* mutation alone might yield a modest association with the *MGMT* single-nucleotide polymorphism. Transition mutations account for 14% of the *PTEN* mutations in glioblastoma multiforme (55) and other glioma-associated transitions also affect the phosphatidylinositol 3′-kinase (PIK3CA) gene (56).

A stronger association and perhaps more novel observation was our finding that glioblastoma multiforme patients whose tumors showed normal TP53 expression (immunohistochemistry and mutation negative) were more likely than population-based controls to carry the putative high activity *MGMT* 84Phe allele. This is potentially biologically significant given that other investigators have suggested, based on the strong interrelationship between MGMT expression and the *TP53* status of cells, that TP53 may regulate MGMT activity (57-60). TP53 wild-type mouse astrocytes showed 5-fold higher levels of MGMT compared with TP53 knockout mice (61). Primary human brain tumors with mutant TP53 were shown to have lower levels of MGMT than nonmutant tumors (62). We hypothesize that under some conditions, normal TP53 expression might selectively promote the survival of cells that carry the putative higher activity *MGMT* 84Phe alleles. This idea posits a pathway of gliomagenesis that would maintain normal TP53. Interestingly, *EGFR* amplification, which is inversely related to *TP53* mutation, was also less common among *MGMT* 84Phe carriers, suggesting that alterations outside both the *EGFR* and *TP53* loci may be relevant in this potential pathway. We found that 46% of glioblastoma multiforme cases showed no abnormalities in the *TP53, EGFR*, or *MDM2* genes. Rodent models of gliomagenesis have suggested one possibility for this finding. DNA ploidy changes associated with overexpression of cyclin-dependent kinase 4 were found in TP53 wild-type and EGFR nonamplified mouse astrocytic tumors (63). Hyperploidy in human glioblastoma multiforme is common (64). Hyperploidy may arise under specific selection conditions when constraints on proliferation and nutrients may make *TP53* mutation less advantageous in clonal evolution (65). The importance of difference in selective pressures determining tumorigenic pathways has recently been emphasized (66). Because these observations are based on relatively small sample size, our conclusions must be considered with some caution. Although the associations of *MGMT* Leu84Phe variants with molecular subtypes of glioblastoma multiforme observed in this study suggest that this polymorphism may be directly involved in the pathogenesis of glioma, linkage of the Leu84Phe variant to other loci affecting MGMT expression or activity is also a possibility.

There may be clinical implications of the interrelationships of *MGMT* genotype, MGMT activity, and *TP53* status of the tumor [i.e., survival differences in glioma patients related to age may in part reflect the fact that younger patients are more likely to have tumors that harbor *TP53* mutations, which are more responsive to 1,3-bis(2-chloroethyl)-1-nitrosourea chemotherapy (61) at least, in part, due to lower MGMT-mediated repair]. Taken together, these observations emphasize the need for further study of individual variations of MGMT activity in the etiology and treatment of adult glioma (67). It will be of future interest to examine germ line MGMT variation with MGMT inactivation via methylation in tumors in association with alterations and expression of TP53 and other glioma tumor suppressor genes.

In conclusion, TP53 and EGFR, the most widely studied molecular features of astrocytic brain tumors in adults, define somewhat overlapping categories of the disease with respect to age at diagnosis, histology, and other characteristics such as race/ethnicity. Choosing the best approach in marking abnormalities in these pathways (i.e., genomic alteration, protein expression, or both) is complicated and a satisfactory algorithm does not yet exist despite strong statistical associations among markers. Given these limitations, however, our observations indicate that age, race/ethnicity, and inherited genetic factors are linked to molecular features, and it seems that applying these markers in molecular epidemiology studies holds promise in searching out the underlying causes of these cancers.

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Acknowledgments

We thank Richard Davis, M.D., for pathology review of series 1 cases and the pathology departments of Alexian Brothers Medical Center, Alta Bates Summit Medical Center, Brookside, California Pacific Medical Center, Doctor's Medical Centers Pinole and San Pablo, Eden Medical Center, El Camino Hospital, Good Samaritan Hospital, Alameda County Medical Center Highland Hospital, John Muir Medical Center, Kaiser Redwood City, Kaiser San Francisco, Kaiser Santa Teresa, Community Hospital of Los Gatos, Los Medano Hospital, Marin General Hospital, Merrithew Memorial Hospital, Mills Peninsula Hospital, Mt. Diablo Medical Center, Mt. Zion Medical Center, Naval Hospital, O'Connor Hospital, Ralph K Davies Medical Center, Saint Louise, San Francisco General, San Jose, San Leandro, San Mateo County, San Ramon Valley, Santa Clara Valley, Sequoia, Seton Medical Center, St. Francis, St. Lukes, St. Rose, Stanford, University of California San Francisco, Valley Livermore Memorial, Veterans Palo Alto, VA Medical Center San Francisco, and Washington Hospital for providing tumor specimens for review and molecular analyses.

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