Positive and Negative Associations of Human Leukocyte Antigen Variants with the Onset and Prognosis of Adult Glioblastoma Multiforme

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Abstract

Associations of genetic factors with malignant gliomas have been modest. We examined the relationships of human leukocyte antigen (HLA) and related polymorphisms to glioblastoma multiforme in adult Caucasians (non–Hispanic Whites) from the San Francisco Bay area. For 155 glioblastoma multiforme patients and 157 control subjects closely matched by ethnicity, age, and gender, PCR-based techniques resolved alleles at *HLA-A*, *-B*, *-C*, and *-DRB1* loci along with short tandem repeat polymorphisms of *MICA* exon 5 and *TNFb*. By multivariable logistic regression, B*13 and the B*07-Cw*07 haplotype were positively associated with glioblastoma multiforme (P = 0.01 and <0.001, respectively), whereas Cw*01 was the only variant showing a negative association (P = 0.05). Among glioblastoma multiforme patients, progression to death after diagnosis was slower in those with A*32 (relative hazard, 0.45; $P < 0.01$) and faster in those with B*55 (relative hazard, 2.27; $P < 0.01$). Thus, both the occurrence and the prognosis of glioblastoma multiforme could be associated with specific but different HLA genotypes. B*07 and the B*07-Cw*07 haplotype are much more common in Caucasians than other ethnic groups in the U.S., which may partially explain the higher incidence of glioblastoma multiforme in Caucasians. (Cancer Epidemiol Biomarkers Prev 2005;14(8):2040–4)

Introduction

Glioblastoma multiforme (also known as WHO II grade IV glioma) is the most common and most severe form of primary malignant brain tumor (1-3). Rare heritable syndromes and high-dose therapeutic radiation have been unequivocally established as the causes of certain glioblastoma multiformes (2, 4); other studies have suggested additional roles for immunologic factors in mediating this molecularly heterogeneous and often rapidly fatal neoplasm (5-8). Ethnic and gender differences in glioblastoma multiforme incidence are also well-documented, with age-adjusted rates of glioblastoma multiforme in the U.S. being 2.5 times higher in Whites (Caucasians) than in blacks (African-Americans) and 60% higher in men than in women between 1990 and 1994 (1, 9-11). Occupational and dietary exposures, infectious agents and other environmental factors have been implicated less consistently (2, 4, 12, 13). Familial associations, linkage studies, as well as studies of single nucleotide polymorphisms in DNA repair, carcinogen metabolism, and cell cycle regulation gradually suggest inherited susceptibility to gliomagenesis (14-26). Overall, it seems likely that genetic, developmental, and environmental factors all contribute to the etiology and pathogenesis of malignant glioma (4).

Human leukocyte antigen (HLA) polymorphisms are known to alter susceptibility to and/or the course of various inflammatory diseases, immune disorders, infectious diseases, and malignancies (27). In humans, both nasopharyngeal carcinoma (28-30) and cervical cancer (31-33) have been convincingly associated with specific HLA alleles and haplotypes. These cancers show strong etiologic relationships to infection with EBV and human papillomavirus, respectively, and the associations may well reflect the important role of HLA molecules in modulating immune responses to infectious agents. Indeed, specific HLA class II haplotypes (e.g., DRB1*1501-DQB1*0602) may differentially influence the risk of cervical neoplasia by directing immune response to HPV16-specific epitopes (34-36). Similarly, HLA-mediated mechanisms may also operate in the pathogenesis of glioblastoma multiforme as human cytomegalovirus infection may facilitate adult gliomas (37, 38).

Several studies have already explored associations between HLA polymorphisms and risk of malignant glioma and related diseases in small cohorts of patients and controls (39-41). HLA and microsatellite typing was first done in two cohorts (n = 42-58) of Japanese and Italian patients (39, 40). In a recent, somewhat larger, study based on molecular HLA typing in 65 glioma cases and 157 controls from Germany (41), the cases showed higher frequencies of A*25, B*18, B*27 and DRB1*15, and a lower frequency of DRB1*07 than controls (41). Differences in results between the Japanese and German studies were quite striking, but so are HLA profiles between Asians and Europeans. We suspected that a larger study of well-categorized glioblastoma multiforme cases and controls, when closely matched by sex, age, ethnicity, and geography, could clarify whether polymorphism at the HLA loci might account for any of the predilection to this neoplasm. Our observations do suggest that variations at class I loci are associated with incidence and variable prognosis of glioblastoma multiforme.

Materials and Methods

Study Subjects. Subjects for this study came from a population-based case-control study of adult glioma in the

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San Francisco Bay area (7, 12, 42, 43). Briefly, malignant glioma patients were eligible for the study if: (*a*) age 20 or older; (*b*) residence (at time of histologically diagnosed glioma) in Alameda, Contra Costa, Marin, San Mateo, San Francisco, or Santa Clara counties; and (*c*) date of diagnosis from August 1991 to April 1994 (series 1) or May 1997 to August 1999 (series 2; refs. 7, 12, 42, 43). Patients were ascertained within a median of 7 weeks of initial diagnosis using Northern California Cancer Center's Rapid Case Ascertainment program (7, 42). For the same study, healthy control subjects were identified and interviewed through random-digit dialing until they had the 1:1 match with the cases by age (±1), gender and ethnicity (7). From the total of 519 glioma cases reviewed by Richard Davis (Department of Pathology, University of California, San Francisco, San Francisco, CA; for series 1) and Kenneth Aldape (Department of Pathology and Brain Tumor Center, M.D. Anderson Cancer Center, Houston, TX; for series 2), 157 with WHO grade IV glioma (glioblastoma multiforme) and 157 controls best matched to cases for age and gender were available for this substudy based on two criteria: being Caucasian (self-identified as White, non-Hispanic) and having sufficient blood specimen for DNA extraction. The original research and this follow-up study conformed to human experimentation guidelines set forth by the U.S. Department of Health and Human Services. Procedures for informed consent and data analyses were approved by institutional review boards at all local and sponsoring organizations.

Whole Genome Amplification and Molecular Genotyping. Genomic DNA was extracted by automated, organic procedures from heparinized blood samples stored at −20°C. Phi29 DNA polymerase and random hexamers were used for whole genome amplification, following protocols recommended by the manufacturer (Amersham Biosciences, Piscataway, NJ), as described in detail elsewhere (44). A combination of PCR-based techniques, including solid-phase sequencing, PCR with sequence-specific primers and automated, reference strand–mediated conformation analyses, resolved individual alleles from the classical HLA genes *HLA-A*, *-B*, *-C*, and *-DRB1* at chromosome 6p21.3 (44, 45). Alleles of the *TNFb* microsatellite [short tandem repeat (STR) sequence] and of another STR in exon 5 of the MHC class I-like chain A (*MICA*) gene were resolved through PCR amplification and automated, denaturing gel electrophoresis (44).

Statistical Analysis. Statistical Analysis Software, version 9.0 (SAS Institute, Cary, NC) was used for descriptive statistics and comparative analyses. First, the distributions (*n* and %) of genetic variants (alleles and haplotypes) were tabulated by direct counting, with the numbers of chromosomes (2*N*) as the denominators. Overall differences in allele frequencies between glioblastoma multiforme cases and controls were assessed in global tests (6,250,000 random simulations; ref. 46); $P \leq 0.05$ signified genetic heterogeneity at a given locus. Second, associations were analyzed for individual alleles found in at least 10 subjects and odds ratio (OR) for glioblastoma multiforme with 95% confidence intervals (CI) were calculated by logistic regression, using the numbers of subjects (*N*) as the denominators. Initial attention was paid to all genetic variants with OR >1.5 or <0.7. Those in tight linkage disequilibrium were evaluated in stratified analyses in order to separate primary from secondary contributions. A P value ≤ 0.05 was accepted as a screening threshold for inclusion in further analysis. Third, independent associations of genetic markers with glioblastoma multiforme were defined by multivariable logistic regression models including age and gender to adjust for residual differences in those variables. A backward, stepwise selection procedure generated the most parsimonious model. Factors with an adjusted (multivariable) P value ≤ 0.05 were accepted as the putative contributors or markers. Fourth, survival patterns for patients with different genotypes were compared in Kaplan-Meier plots. Estimates of hazards ratios for death and 95% CI were based on the Cox proportional hazards model. The P values shown throughout the following section were not corrected for multiple comparisons. Instead, observed associations were tested for internal and external consistencies.

Results

PCR-based genotyping was successful for all six target loci (*HLA-A*, *-B*, *-C*, *-DRB1*, *MICA* exon 5 STR, and *TNFb*) in 153 (97%) out of 157 Caucasian glioblastoma multiforme cases and in 154 (98%) of 157 control subjects. Two cases (1.5%) had to be excluded for their lack of reliable genotyping results at multiple loci, whereas two more cases and three controls could be analyzed for five of the six target loci.

The 155 cases and 157 controls included in genetic association analyses had respective ages (mean ± SE) of 58.4 ± 0.9 and 58.2 ± 1.0 years and respective sex ratios (M/F) of 1.72 (98/57) and 1.62 (97/60) ($P > 0.50$ for both comparisons). These minor differences in age and gender were treated as covariates in all subsequent comparative analyses, especially because two glioblastoma multiforme cases were not available for such analyses.

Overall Distribution of HLA and Related Markers. At the two-digit specificity level, 14 *HLA-A* variants (A*01, *02, *03, *11, *23, *24, *25, *26, *29, *30, *31, *32, *33, *66 and *68), 17 *HLA-B* variants (B*07, *08, *13, *14, *15, *18, *27, *35, *37, *38, *40, *44, *49, *51, *52, *55 and *57), 12 *HLA-C* variants (Cw*01, *02, *03, *04, *05, *06, *07, *08, *12, *14, *15 and *16), and 11 *DRB1* variants (DRB1*01, *03, *04, *07, *08, *11, *12, *13, *14, *15 and *16) were found in 10 or more subjects, so were 5 *MICA* exon 5 STR alleles (A4, A5, A5.1, A6 and A9). *TNFb* microsatellite typing revealed all seven known alleles; four (b1, b3, b4 and b5) of them were common (allele frequencies ranging from 0.11 to 0.41).

The allelic and genotypic profiles at all six loci conformed to Hardy-Weinberg equilibrium and they also closely resembled those observed in general Caucasian populations in the U.S. (47-49) and Europe (50). Patterns of linkage disequilibrium frequently seen in Caucasians were also confirmed (data available from J. Tang); 18 common B-C haplotypes (B*07-Cw*07, B*08-Cw*07, B*14-Cw*08, B*15-Cw*03, B*18-Cw*05, B*18-Cw*07, B*27-Cw*02, B*35-Cw*04, B*37-Cw*06, B*38-Cw*12, B*40-Cw*03, B*44-Cw*05, B*44-Cw*07, B*44-Cw*16, B*49-Cw*07, B*51-Cw*15, B*55-Cw*03 and B*57-Cw*06) could be assigned. The *HLA-B* and *MICA* exon 5 STR haplotypes also contained most of major *HLA-B* alleles (B*07, B*14, B*15, B*18, B*27, B*35, B*44, B*51, B*55 and B*57). For example, HLA-B*07 was in linkage disequilibrium with *MICA* exon 5 STR allele A5.1, which encodes a truncated MICA protein not expressed on cell surface.

Global tests for heterogeneity of major HLA, *MICA* and *TNFb* alleles between the glioblastoma multiforme patients and controls revealed a clear difference for *HLA-B* ($P = 0.05$) but not for any other loci ($P = 0.094$-0.897). Tests of the major HLA-B-C haplotypes yielded a P value of 0.199.

Analyses of Markers Implicated in Earlier Studies. At least nine markers (Table 1) from the target loci had been implicated in earlier studies of small cohorts in Japan, Germany, and Italy (39-41). These markers are relatively common in the glioblastoma multiforme cases and controls studied here, with frequencies ranging from 4.5% to 25.0%. However, none of the earlier associations could be unambiguously confirmed. For example, A*24 and DRB1*15 were previously associated with malignant glioma in Japanese and Germans, respectively, but their distribution in glioblastoma multiforme patients and controls in our study was almost identical (15.5% versus 15.9% for A*24 and 25.2% versus 24.8% for DRB1*15). *TNFb* allele 4 (b4) homozygosity was slightly enriched in glioblastoma

Table 1. Analyses of human leukocyte antigen (HLA) and *TNFb* microsatellite variants previously associated with malignant glioma and related diseases in various cohorts

Genetic variants	Reported association	Distribution, {*n* (%)} in:		OR	*P**
		155 Cases	157 Controls		
A24†	Positive	24 (15.5)	25 (15.9)	0.97	0.92
A*25‡	Positive	6 (3.9)	8 (5.1)	0.75	0.60
B*27‡	Positive	11 (7.1)	11 (7.0)	1.01	0.97
DRB1*15‡	Positive	39 (25.2)	39 (24.8)	1.02	0.95
DRB1*07‡	Negative	34 (21.9)	38 (24.2)	0.88	0.63
Cw*06-DRB1*07‡	Negative	13 (8.4)	11 (7.0)	1.22	0.65
TNFb allele 4 (b4) hmz§	Positive	33 (21.3)	23 (14.6)	1.58	0.13

*Based on maximum likelihood Chi-square tests.
†From a study of 42 Japanese patients (39).
‡From a study of 65 German patients (41).
§Hmz, homozygosity, from a study of 48 Italian patients (40).

multiforme cases (21.3%) compared with controls (14.6%; OR, 1.58; 95% CI, 0.88-2.83; $P = 0.13$); this relationship was somewhat consistent with a positive association seen in Italians.

Positive and Negative Associations of HLA Variants with the Occurrence of Adult Glioblastoma Multiforme: Univariate and Multivariable Analyses. At nominal, univariate P value of ≤ 0.05, five HLA markers showed differential distribution between glioblastoma multiforme patients and controls (Table 2). Individual variants (B*07 and B*13) positively associated with glioblastoma multiforme had univariate ORs ranging from 2.56 to 5.34. The relationship of B*07 to glioblastoma multiforme was less strong than that of the common B*07-Cw*07 haplotype (univariate OR, 2.65; 95% CI, 1.49-4.71). Analyses of B*07 without Cw*07 (very rare), Cw*07 alone (OR, 1.47; $P = 0.09$) and Cw*07 without B*07 (OR < 1.20; $P > 0.50$) confirmed the primary effect of the B*07-Cw*07 haplotype. On the other hand, it was less clear whether the B*13-Cw*06 haplotype association (OR, 4.78; $P = 0.035$) was more likely due to B*13 alone because only a single B*13-positive subject did not have Cw*06; however, neither Cw*06 overall (OR, 1.49; $P = 0.20$) nor Cw*06 without B*13 (OR < 1.20; $P > 0.50$) was associated with glioblastoma multiforme. Meanwhile, only Cw*01 showed a statistically significant negative association with glioblastoma multiforme (OR, 0.34; 95% CI, 0.12-0.97; Table 2). Statistical adjustments for age and gender did not alter the univariate estimates of ORs and P values in any significant way (data available from J. Tang).

In multivariable analysis, three HLA markers (B*07-Cw*07, B*13, and Cw*01) most likely accounting for the above observations were all independently associated with glioblastoma multiforme (Table 3, model 1). These associations did not change appreciably after further adjustment for age and gender (Table 3, model 2). In short, the B*07-Cw*07 haplotype and B*13 were independently associated with glioblastoma multiforme (adjusted OR, 2.78 and 7.53; 95% CI, 1.55-4.98 and 1.56-36.31, respectively), whereas Cw*01 was negatively associated (OR, 0.33; 95% CI, 0.11-0.99). Association of the B*07-Cw*07 haplotype with glioblastoma multiforme was the only one that could withstand Bonferroni correction for multiple comparisons: the P value became <0.02 when multiplied by 18 (the number of major haplotypes tested).

Survival Analyses of Glioblastoma Multiforme Cases Alone. The 155 glioblastoma multiforme patients had a median survival of 13 months (interquartile range, 8-18 months). In Kaplan-Meier analyses, 9 A*32- and 12 B*55-positive glioblastoma multiforme patients differed from those carrying other alleles in their progression to death after diagnosis (log-rank and Wilcoxon $P < 0.01$ for both). In a combined analyses with all other patients as the referent group (Fig. 1), the relative hazards for death and 95% CIs were 0.40 (0.20-0.79) for A*32 and 2.26 (1.23-4.15) for B*55 ($P < 0.01$ for both). No patient had the combination of A*32 and B*55. Statistical adjustments for residual differences in age and gender did not alter the relationships (adjusted relative hazards, 0.43; $P = 0.018$ for A*32 and relative hazards, 2.28; $P = 0.008$ for B*55). Several other variants, including B*41-Cw*17, B*51-Cw*12, B*57-Cw*07, and DRB1*14 also showed trends toward association with disease prognosis (univariate $P \leq 0.05$), but in each case, either too few subjects were available to permit strong inference or the association disappeared in multivariable analyses (data not shown).

Alternative Association Analyses. Several alternative analyses were also done. First, a number of *HLA-A*, *-B*, and *-DRB1* alleles resolved to four digits (e.g., B*4402, *4403, DRB1*1101, *1102, *1301, and *1302) were common enough for separate comparisons. Second, overall homozygosity at each locus or homozygosity of individual variants was compared between glioblastoma multiforme patients and controls. Third, *HLA-A* and *HLA-B* alleles were analyzed according to their known supertypes that share similarities in peptide-binding

Table 2. HLA markers provisionally associated with adult glioblastoma multiforme (GBA) through univariate analyses

HLA marker*	Distribution, {*n* (%)} in:		OR (95% CI)	Univariate *P*†
	155 Cases	157 Controls		
HLA-B*07	49 (31.6)	24 (15.3)	2.56 (1.48-4.44)	<0.001
HLA-B*13	10 (6.5)	2 (1.3)	5.34 (1.15-24.80)	0.020‡
HLA-Cw*01	5 (3.2)	14 (8.9)	0.34 (0.12-0.97)	0.044
B*07-Cw*07	45 (29.0)	21 (13.4)	2.65 (1.49-4.71)	<0.001
B*13-Cw*06	9 (5.8)	2 (1.3)	4.78 (1.02-22.48)	0.035‡

*Alleles and haplotypes tentatively associated with GBM, based on nominal $P \leq 0.05$. OR, odds ratio; CI, confidence interval. Note that neither Cw*07 alone nor Cw*06 alone is associated with GBM (see text).
†Based on maximum likelihood Chi-Square test unless specified otherwise.
‡By 2-sided, Fisher's exact test.

Table 3. HLA markers independently associated with adult glioblastoma multiforme in multivariable analyses

HLA factors*	Model 1†		Model 2†	
	OR (95% CI)	*P*	OR (95% CI)	*P*
B*07-Cw*07	2.79 (1.56-5.00)	<0.001	2.78 (1.55-4.98)	<0.001
HLA-B*13	7.07 (1.49-33.57)	0.014	7.53 (1.56-36.31)	0.012
HLA-Cw*01	0.35 (0.12-1.03)	0.057	0.33 (0.11-0.99)	0.047

*Initially identified through univariate analyses (Table 2).
†Model 1 includes all independent markers (i.e., those not explained by linkage disequilibrium); model 2 reflects further adjustment for age and gender.

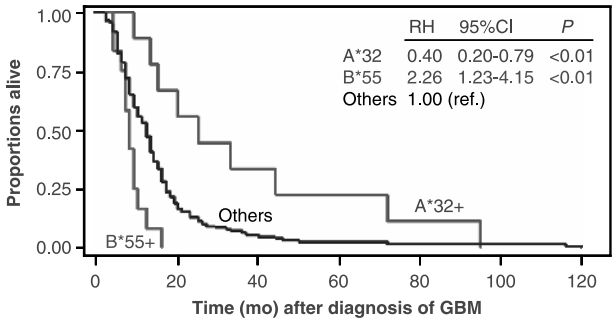

Figure 1. Progression to death among adult glioblastoma multiforme patients carrying HLA variants individually associated with faster or slower progression (log-rank $P < 0.001$ and Wilcoxon $P = 0.001$). Relative hazards (RH) for death and 95% CIs as shown for A*32 and B*55 are based on the Cox proportional hazards model, in which patients without either variants form the reference (ref.) group (proportionality test $P = 0.14$).

preferences (51, 52). Fourth, *HLA-DRB1* alleles were grouped into five lineages (DR1, DR51, DR52, DR53, and DR8) corresponding to local linkage disequilibrium (and gene contents) with the neighboring genes *HLA-DRB3*, *-DRB4*, and *-DRB5* (27, 53). Fifth, common extended haplotypes from *HLA-A* to *DRB1* (e.g., A*01-Cw*07-B*08-DRB1*03) were evaluated by counting individuals with combinations of all alleles known to form extended haplotypes across the HLA blocks. Sixth, pairs (diplotypes) of HLA (e.g., B*07/B*08 and B*07/B*44), *MICA* exon 5 (e.g., A4/A5 and A4/A9) and *TNFb* alleles (e.g., b1/b3 and b1/b4) were treated as unique entities for comparisons. Seventh, association analyses were done separately in patients and controls stratified by gender. To summarize these analyses, *HLA-DRB1* homozygosity (identity of paternal and maternal alleles at the four-digit level) showed a trend toward a negative association with glioblastoma multiforme (OR, 0.49; 95% CI, 0.23-1.02; $P = 0.06$), but this relationship diminished in multivariable analysis. Among the extended haplotypes, A*03-Cw*07-B*07-DRB1*15 had a slightly higher frequency in cases (univariate OR, 1.63; 95% CI, 0.69-3.89; $P = 0.27$). In every other comparison, the P value was >0.30. Finally, stratified analyses did not reveal any additional gender-specific associations.

Discussion

Human MHC (HLA) genes are the most polymorphic within the human genome (27, 54). The large number of genotypes (alleles and haplotypes) defining these loci have been presumably maintained primarily through balancing selection by a variety of human diseases. Recent discoveries (37, 38) that human cytomegalovirus infection might play some role in the pathogenesis of adult gliomas make *HLA* genes highly relevant as candidates for association with this disease. Our systematic analyses of HLA and related variants (alleles, haplotypes, supertypes, lineages, diplotypes, and homozygosity) at chromosome 6p21.3 revealed three HLA markers independently associated (two positively and one negatively) with adult glioblastoma multiforme. These markers lie centromeric to the *HLA-A* locus and telomeric to the *HLA-DRB1* locus, suggesting that *HLA-B*, *HLA-C* and perhaps other genes in the central MHC (HLA class III and class IV) region can mediate the occurrence of adult glioblastoma multiforme. The lack of a relationship to *MICA* exon 5 STR alleles tentatively dismissed this HLA class I-like locus, along with other loci centromeric to *HLA-B*, as likely contributors to the observed effect.

In global tests, the overall distribution of *HLA-B* locus alleles showed statistically significant heterogeneity between our glioblastoma multiforme patients and controls, whereas statistically significant associations of individual alleles were likewise confined to those at the adjacent *HLA-B* and *HLA-C* loci. The positive association of the B*07-Cw*07 haplotype with glioblastoma multiforme was of particular interest because this association remained statistically significant after correction for the number of major haplotypes tested in our study and both B*07 and Cw*07 were also enriched in certain German patients with glioblastoma multiforme (41). Other HLA variants at loci on either side of the *HLA-B* to *HLA-C* segment, i.e., A*03, DRB1*15 and *MICA* exon 5 allele A5.1, which are known to form extended haplotypes with B*07-Cw*07, did not show appreciable relationships with glioblastoma multiforme, suggesting that the association of B*07-Cw*07 did not arise from apparent linkage disequilibrium with variants at the other genotyped loci.

The biological significance of the association with the B*07-Cw*07 haplotype is not obvious. Because the majority of genes in the central MHC region participate in CTL response, natural killer function, and inflammation (27), the association most likely reflects involvement of some combination of those functions. Increased presence of B*07-Cw*07 among Caucasian patients with glioblastoma multiforme compared with controls may also help explain the higher incidence in persons of European ancestry (1, 11). The higher frequency of this haplotype in Caucasians than other ethnic groups in the U.S. has been well-established (47).

The two HLA variants (A*32 and B*55) associated with the prognosis of glioblastoma multiforme were relatively uncommon in the study population. In the absence of a clear biological mechanism for the involvement of either, these modest associations should be interpreted just as cautiously as others reported in small cohorts (39-41). Among the eight markers already proposed by other investigators, only *TNFb* allele 4 (b4) homozygosity showed a slight trend toward positive association with adult glioblastoma multiforme in our investigation. *TNFb* microsatellite alleles located in the central MHC region frequently mark common haplotypes that contain single nucleotide polymorphisms at the loci encoding tumor necrosis factor-α, lymphotoxin-α, and lymphotoxin-β. A formal evaluation of functional single nucleotide polymorphisms at these and other related loci may prove informative.

Genes in the HLA region are not the only ones of interest for glioblastoma multiforme. For example, several markers in genes participating in various pathways not immediately related to immune responses (e.g., *CYP2E1*, *GSTP1*,and *GSTT1*) have been implicated in malignant glioma (15, 23, 43, 55). Although differences among studies in the application of histopathologic classification systems and selection of particular patients for genotyping may preclude direct comparison of research findings, availability of glioblastoma multiforme patients and controls with defined HLA genotypes should provide a useful foundation on which to further explore or refine the nature and extent of multigenic influences on malignant brain tumors.

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