Cytochrome *P*450 1B1 Expression in Glial Cell Tumors: An Immunotherapeutic Target

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Abstract

Purpose: Among central nervous system malignancies, cytochrome *P*450 1B1 (CYP1B1) expression has only been characterized in medulloblastoma. An immunotherapeutic agent targeting this antigen was shown to safely stimulate a good immune response. To evaluate the viability of further research efforts targeting this antigen, we examined the expression of CYP1B1 in glial cell malignancies.

Experimental Design: We studied the frequency and extent of CYP1B1 expression by immunohistochemical analysis in 269 glial tumors (including all major pathologic types) on a tissue microarray. Results were categorized by percentage of cells stained and intensity of cytoplasmic staining within cells. Correlation of CYP1B1 expression with patient prognosis was evaluated by univariate and multivariate analyses.

Results: Overall, increased CYP1B1 expression in glial tumors was associated with decreased patient survival time ($P < 0.0014$ for both percentage and intensity of staining). A significant difference existed in percentage and intensity of staining between astrocytic and oligodendroglial tumors ($P = 0.0002$ and 0.0003, respectively), between grades of tumors ($P < 0.0001$ and 0.0079), and between pathologic types of tumors ($P < 0.0001$ and 0.0339). Positive CYP1B1 staining was seen in 81% of glioblastomas, 84% of anaplastic astrocytomas, 61% of oligodendrogliomas, and 67% of anaplastic oligodendrogliomas. Paradoxically, within specific tumor pathologies, there was a trend toward increased survival as CYP1B1 expression increased. However, in the multivariate analysis, this trend disappeared, and CYP1B1 expression seemed prognostically neutral.

Conclusion: CYP1B1 is frequently expressed in a variety of gliomas and could be used as a target for immunotherapy.

Cytochrome *P*450 1B1 (CYP1B1) is a tumor-associated antigen that is overexpressed in a wide range of hematologic malignancies and solid tumors (1). Although CYP1B1 is expressed in normal tissues, such as the breast and prostate (2, 3), it is expressed at much higher levels in tumor cells compared with the surrounding normal tissues (2). Both normal human astrocytes and neurons have been reported to express CYP1B1 (3), but this expression is confined to the cell nucleus based on immunohistochemistry. Among central nervous system malignancies, expression of CYP1B1 has only been shown in human medulloblastoma cells (4). The CYP1B1 antigen has been exploited as an immune target in a phase I clinical trial. Among patients with solid and hematologic tumors treated with a plasmid vector expressing the CYP1B1 antigen encapsulated in poly(DL-lactide-coglycolide) microparticles (in whom several prior therapies had failed), patients in which an anti-CYP1B1 immune response could be detected exhibited objective clinical responses without evidence of induced autoimmunity, suggesting that CYP1B1 represents a viable target for cancer immunotherapy (5). Alternatively, under hypoxic tumor conditions, CYP1B1 can convert the drug AQ4N (a topoisomerase inhibitor) to the cytotoxic amine AQ4, indicating a possible role for this novel chemotherapeutic agent in glioma patients (6, 7).

The identification of novel immunologic targets for glioma therapy is critical for a variety of reasons. Gliomas, especially those of high grade, are not particularly responsive to conventional therapies, such as radiation therapy and chemotherapy. However, several recent immunotherapy clinical trials treating high-grade glioma patients have shown promising results (8). For example, when glioma patients were vaccinated with dendritic cells and acid-eluted tumor peptides (9), dendritic cells and a peptide spanning the tumor-specific antigen epidermal growth factor variant III (10), or a peptide vaccine directed against epidermal growth factor variant III

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(11), an immune response was induced and survival time was enhanced, indicating that immunotherapy may be a viable treatment option for these patients. Second, although other antigens that could be exploited as immunotherapeutic targets have been identified, such as Tie2, tenascin-C, survivin, Sart-1, and MAGE-1, many of these are suboptimal, owing to the lack of universal expression, lack of cell surface expression, and MHC restriction. Furthermore, there is a paucity of targets for gliomas other than glioblastoma multiforme, which is unfortunate because the longer survival interval in these patients and the less inherent immunosuppression would provide an improved opportunity for the activation of immune response. Finally, the identification of alternative immune targets will be necessary because treatment failure can arise secondary to the presence of antigen escape variants (12).

In this study, we determined the expression of CYP1B1 in a variety of glial tumors, including those of low grade. Additionally, we evaluated whether the expression of CYP1B1 was correlated with survival among and within glioma subtypes and WHO tumor grades. Using multivariate analysis, the effect of CYP1B1 was assessed as a prognostic factor. We found that CYP1B1 may be a viable immunotherapeutic target for gliomas and that it is prognostically neutral as a tumor marker.

Materials and Methods

Clinical samples. A tissue microarray was used, which included tissue from 73 glioblastomas (grade 4), 46 anaplastic astrocytomas (grade 3), 9 low-grade astrocytomas (grade 2), 44 oligodendrogliomas (grade 2), 36 anaplastic oligodendrogliomas (grade 3), 16 mixed oligoastrocytomas (grade 2), 23 anaplastic mixed oligoastrocytomas (grade 3), and 9 gliosarcomas (grade 4), which was assembled by the study neuropathologist (G.N.F.) with the tumor pathology confirmed by the final issued report at The University of Texas M. D. Anderson Cancer Center and archived in paraffin blocks. The microarray, as described previously (13), also contained normal brain tissue (white matter, cortex, and cerebellum) and glioma cell lines. Normal brain tissue was obtained from parenchyma that overlaid deep metastases from surgical specimens that were not involved by neoplasm. No autopsy tissue was used as normal brain. The glioma cell lines are included as nonspecific controls for antigen integrity. The extent of surgical resection was defined as the amount of volumetric resection of the contrast-enhancing component on the preoperative T1-weighed magnetic resonance imaging imaged as analyzed by Vitrea 3 software as described previously (14). The only tumors in the array that contained recurrent specimens were the glioblastoma multiforme group (n = 12), which was insufficient to draw conclusions about the effects of treatment on CYP1B1 expression. We also did a supplemental analysis without data from the reoperation cases, with no significant change in the results. This study was conducted under the Institutional Review Board–approved protocol LAB03-0228 at The University of Texas M. D. Anderson Cancer Center.

Immunohistochemistry. Immunohistochemistry for CYP1B1 was carried out using a Dako autostainer (DakoCytomation) as described previously (15). Sections (*μm) of the brain tumor microarray were dewaxed in xylene and rehydrated in ethanol, and an antigen retrieval step was done. The antigen retrieval step consisted of microwaving the sections in 0.01 mol/L citrate buffer at pH 6.0 for 20 min in an 800 W microwave oven operated at full power. The sections were then allowed to cool to room temperature. Monoclonal anti-human CYP1B1 (16) antibody diluted in antibody diluent (Dako) was applied for 60 min at room temperature and washed with buffer (Dako) followed by peroxidase blocking for 5 min (Dako) followed by a single 2-min buffer wash. Prediluted peroxidase-polymer–labeled goat anti-mouse/rabbit secondary antibody (EnVision, Dako) was applied for 30 min at room temperature followed by further washing with buffer to remove unbound antibody. Sites of peroxidase activity were then shown with diaminobenzidine as the chromogen applied for two successive 5-min periods. Sections were washed in tap water, lightly counterstained with hematoxylin, and enhanced in 0.5% copper sulfate solution. Finally, the sections were then dehydrated and mounted in a synthetic mounting medium. Sections of an ovarian cancer known to express CYP1B1 were used as a positive control. Omitting the primary antibody from the immunohistochemical procedure and replacing it with antibody diluent acted as negative control (13).

Each sample, in duplicate, within the array was examined under the microscope by three independent observers (J.A.B., A.B.H., and G.N.F.) in blinded fashion, and the presence or absence of staining was evaluated. Tumors displaying cytoplasmic staining in 1% or more of cells were judged to be positive. The cutoff of 1% was the level in which all three independent observers were convinced that there was staining above background and to eliminate the possibility of minor artificial staining. In the case of negative staining (<1%), a value of 0 was recorded. In the case of positive staining, the percentage of cells and the intensity of staining were judged independently of each other, and the results were recorded. The percentage of cells showing expression was graded as 1 (1-50%) or 2 (>50%). Intensity of staining was graded as 1 (low) or 2 (strong). Our neuropathologist (G.N.F.) ascertained that there was a demarcation into what seemed to be low and high staining as opposed to a graduated or Gaussian distribution. Potential mismatching of data was minimized by staining an intact microarray with H&E and identifying the correct location of each tissue core by visually matching the tumors based on their unique histologic elements.

Statistical analysis. Initially, there were specimens from 269 patients on the tissue microarray. Ten patients were omitted from the survival analyses because their microarray specimens consisted of normal brain tissue. Two patients were omitted because their tumor pathology could not be independently confirmed. An additional 48 patients were omitted for the following reasons: (a) we could not conclusively determine tumor staining results, (b) the position of the tumor sample on the array was indeterminate, (c) the sample was lost during slide processing, or (d) the patient was lost to follow-up. This left 209 patients whose samples were used to determine the effect of CYP1B1 expression on survival. There were eight patients who were reported deceased but whose dates of death were unknown. For the purposes of this analysis, these patients were considered alive at the date of last contact and lost to follow-up thereafter.

A univariate analysis was initially conducted to determine the relationship of staining, glial tumor category (pure astrocytic versus oligoastrocytic and mixed oligoastrocytic), tumor grade, specific tumor pathology, patient survival time, or patient age, respectively, to the percentage of cells stained and staining intensity. Contingency tables were created to examine the distribution of tumor grade, specific tumor pathology, and glial tumor type in relation to percentage of cells stained and staining intensity. Fisher's exact test was used to determine whether the relationship between these variables was statistically significant. An ANOVA model was then used to determine whether differences in patients' ages correlated with either percentage of tumor cell staining or staining intensity. A Cochran-Mantel-Haenszel test was calculated to examine the relationship between either staining or intensity and glial tumor type while adjusting for tumor grade.

The effect of glial tumor category, tumor grade, specific tumor pathology, percentage of cell staining, and intensity of stain on mortality was examined using Kaplan-Meier survival analysis. Median time to death was calculated, and a log-rank test was computed to determine whether differences in survival time existed. A multivariate analysis to account for known prognostic factors, such as age, tumor type, and performance status was then done on these results for tumor categories in which a strong trend affecting survival seemed to exist.

Results

Study population. The median age of the patients was 42 years (range, 4-91 years). The majority of patients (97%) had a Karnofsky performance scale (KPS) score of ≥70. Seven patients had undergone a biopsy. Among the glioblastoma multiforme patients, the median extent of resection was 96%. Table 1 summarizes the patients' demographic characteristics, and they do not differ significantly from those seen in previous studies.

The percentage of cells expressing CYP1B1 varies according to grade of tumor. As the glioma grade increased, the percentage of cells that stained positively for CYP1B1 expression also increased (Table 2). Because astrocytomas include higher-grade tumors (WHO IV) than oligodendrogliomas, a Cochran-Mantel-Haenszel test was used to determine the relationship between tumor and percentage of cells stained while adjusting for grade. When the adjustment for grade is made, the relationship between glial tumor type and number of cells stained is not statistically significant ($P = 0.1906$). The 1p/19q deletion did not correlate with enhanced CYP1B1 expression in tumors of oligodendroglial origin ($n = 5$; data not shown).

The percentage of cells expressing CYP1B1 varies according to specific tumor pathology. To examine the differences in specific tumor pathology independently of differences stemming from astrocytic or oligodendroglial lineages, the relationship between specific tumor pathology and staining was examined (Table 2). Fisher's exact test showed that, for astrocytomas, the percentage of cells stained was related to specific pathology ($P = 0.002$) but that this relationship did not hold for oligodendroglial tumors ($P = 0.71$). The differences in percentages of cells stained within astrocytic-specific tumors generally fell in line with the pathologic grade, but the relationship was not as linear as the overall relationship between grade and staining intensity. Of note, all of the gliosarcomas that subsequently developed into glioblastomas were among the samples with significant staining ($n = 3$). As might be expected, the percentage of cells staining in most anaplastic astrocytomas (WHO III) was between 1% and 50%, but the percentage of cells staining in low-grade astrocytomas (WHO II) was evenly distributed between no staining and 1% to 50% of cells stained. However, it should be noted that low-grade astrocytomas are rare in adults and this is a small sample number. Further subdivisions based on the percentage of cell staining did not yield statistically significant results.

The intensity of staining of CYP1B1 varies according to grade of tumor. To determine the relationship of staining intensity to increasing tumor grade, the tumors were characterized based on no staining, light to moderate staining, or strong staining. In contrast to the results for percentage of cells stained, based on a Cochran-Mantel-Haenszel test that evaluated the relationship between tumor and the intensity of staining while adjusting for grade, a relationship was seen between tumor type and intensity of stain ($P = 0.0316$).

The intensity of staining of CYP1B1 depends on glial tumor type. To determine whether differences observed for CYP1B1 staining in different tumors were characteristic of the specific pathologic subtypes diagnosed or a result of basic differences in glial tumor types (i.e., astrocytic versus oligodendroglial), a

Table 1. Distribution of characteristics of patients with glial tumors according to tumor classification

Characteristic*	Tumor classification			
	GBM	**GS**		**All grade IV**
No. patients	63	8		71
Median age, y (range)	52 (17-77)	54.5 (23-68)		52 (17-77)
Median KPS (range)	90 (60-100)	80 (60-100)		90 (60-100)
Median survival, mo (range)	14.1 (0.6-43.0)	5.4 (2.9-22.3)		14.0 (0.6-43.0)
	AA	**AO**	**AMOA**	**All grade III**
No. patients	43	21	18	82
Median age, y (range)	43 (4-91)	40 (10-59)	35 (22-52)	40 (4-91)
Median KPS (range)	90 (60-100)	90 (70-100)	95 (70-100)	90 (60-100)
Median survival, mo (range)	27.7 (1.1-56.4)	62.1 (1.7-62.1)	89.2 (8.0-89.2)	33.9 (1.1-89.2)
	LGA	**O**	**MOA**	**All grade II**
No. patients	7	37	12	56
Median age, y (range)	32 (4-47)	39 (7-60)	31.5 (21-57)	37.5 (4-60)
Median KPS (range)	95 (80-100)	100 (70-100)	100 (60-100)	100 (60-100)
Median survival, mo (range)	NA (48.3-48.3)	99.8 (8.5-99.8)	NA (56.1-56.1)	NA (8.5-99.8)
	All astrocytomas	**All Os**		**All tumors**
No. patients	121	88		209
Median age, y (range)	46 (4-91)	38.5 (7-60)		41 (4-91)
Median KPS (range)	90 (60-100)	100 (60-100)		90 (60-100)
Median survival, mo (range)	18.8 (0.6-56.4)	99.8 (1.7-99.8)		41.8 (0.6-99.8)

Abbreviations: GBM, glioblastoma multiforme; GS, gliosarcoma; AA, anaplastic astrocytoma; AO, anaplastic oligodendroglioma; AMOA, anaplastic mixed oligoastrocytoma; LGA, low-grade astrocytoma; O, oligodendroglioma; MOA, mixed oligoastrocytoma; NA, not available.
*Calculations include only patients for whom staining data were available.

Table 2. Distribution of tumor characteristics according to number of cells stained

	No. cells stained			P*
	<1% (*n* = 65)	1-50% (*n* = 78)	>50% (*n* = 66)	
Grade, *n* (%)				
2	26 (40.0)	23 (29.5)	7 (10.6)	<0.0001
3	24 (36.9)	37 (47.4)	21 (31.8)	
4	15 (23.1)	18 (23.1)	38 (57.6)	
Glial tumor type, *n* (%)				
Astrocytoma	26 (40.0)	45 (57.7)	50 (75.8)	0.0002
Oligodendroglial	39 (60.0)	33 (42.3)	16 (24.2)	
Pathologic classification, *n* (%)				
GBM	12 (18.5)	15 (19.2)	36 (54.5)	<0.0001
GS	3 (4.6)	3 (3.8)	2 (3.0)	
AA	8 (12.3)	24 (30.8)	11 (16.7)	
LGA	3 (4.6)	3 (3.8)	1 (1.5)	
AO	8 (12.3)	7 (9.0)	6 (9.1)	
AMOA	8 (12.3)	6 (7.7)	4 (6.1)	
O	16 (24.6)	16 (20.5)	5 (7.6)	
MOA	7 (10.8)	4 (5.1)	1 (1.5)	
Age of patient, y				
Mean (SD)	40.5 (13.3)	39.2 (13.6)	48.3 (14.9)	0.0003

*From Fisher's exact test for grade, glial tumor type, and pathologic classification; from ANOVA for patient's age.

Fisher's exact test was calculated to assess the relationship between pathologic classification and staining intensity for general and specific tumor classes (Table 3). The relationship between pathologic classification and glial tumor type does not hold for astrocytomas ($P = 0.38$) nor for oligodendroglial tumors ($P = 0.90$), thus indicating that the statistical significance of the relationship between specific tumor pathology and staining intensity is driven by glial tumor lineage and not by pathologic subtype. In other words, the significant difference in staining intensity observed between specific pathologies (glioblastoma, anaplastic astrocytoma, etc.) occurs because astrocytic tumors stain more intensely than oligodendroglial tumors (Figs. 1 and 2) and also tend to behave in a more malignant fashion, which results in their being given higher WHO grades.

The percentage of cells expressing CYP1B1, but not the intensity of its expression, varies with the age of the patient. The percentage of cells staining positively for CYP1B1 increased with the patient's age ($P = 0.0003$; Table 2). The mean age of patients with tumors showing <1% or between 1% and 50% of cells stained was 40.5 (SD, 13.3) years and 39.2 (SD, 13.6) years, respectively. The mean age of patients with tumors showing >50% of cells stained was 48.3 (SD, 14.9) years. In contrast, no difference with respect to age was identified in

Table 3. Distribution of tumor characteristics according to intensity of staining

	Intensity of staining			P*
	None (*n* = 65)	Light to moderate (*n* = 93)	Strong (*n* = 51)	
Grade, *n* (%)				
2	26 (40.0)	24 (25.8)	6 (11.8)	0.0079
3	24 (36.9)	37 (39.8)	21 (41.2)	
4	15 (23.1)	32 (34.4)	24 (47.1)	
Glial tumor type, *n* (%)				
Astrocytoma	26 (40.0)	56 (60.2)	39 (76.5)	0.0003
Oligodendroglial	39 (60.0)	37 (39.8)	12 (23.5)	
Pathologic classification, *n* (%)				
GBM	12 (18.5)	31 (33.3)	20 (39.2)	0.0339
GS	3 (4.6)	1 (1.1)	4 (7.8)	
AA	8 (12.3)	21 (22.6)	14 (27.5)	
LGA	3 (4.6)	3 (3.2)	1 (2.0)	
AO	8 (12.3)	9 (9.7)	4 (7.8)	
AMOA	8 (12.3)	7 (7.5)	3 (5.9)	
O	16 (24.6)	17 (18.3)	4 (7.8)	
MOA	7 (10.8)	4 (4.3)	1 (2.0)	
Age of patient, y				
Mean (SD)	40.5 (13.3)	42.9 (14.2)	44.2 (16.1)	0.3714

*From Fisher's exact test for grade, glial tumor type, and pathologic classification; from ANOVA for patient's age.

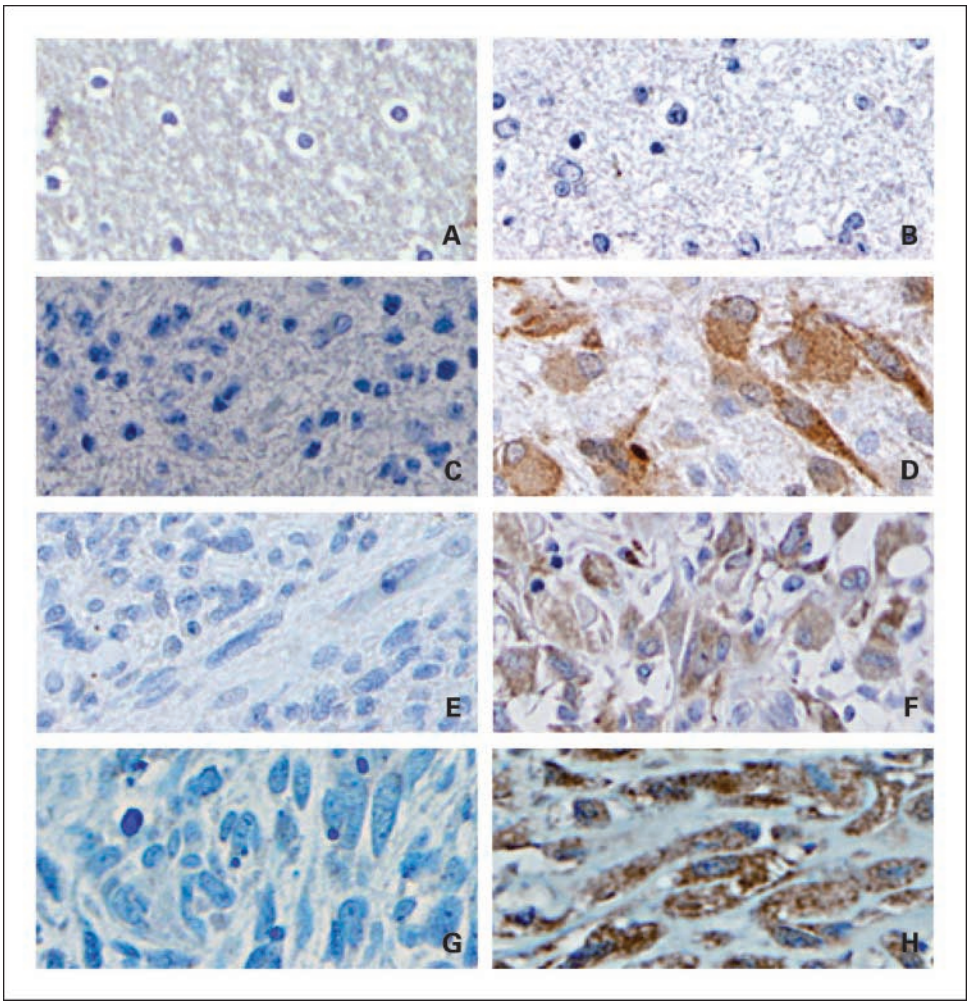

Fig. 1. Photomicrographs of staining results for astrocytomas and normal brain. Magnification, ×200. *A,* normal brain cortex showing negative staining of CYP1B1. *B,* low-grade astrocytoma showing negative staining of CYP1B1. *C,* anaplastic astrocytoma showing negative staining of CYP1B1. *D,* anaplastic astrocytoma showing positive staining of CYP1B1. *E,* glioblastoma showing negative staining of CYP1B1. *F,* glioblastoma showing positive staining of CYP1B1. *G,* a gliosarcoma showing negative staining of CYP1B1. *H,* a gliosarcoma showing positive staining of CYP1B1.

the distribution of intensity of CYP1B1 staining (P = 0.3714; Table 3).

CYP1B1 expression is a prognostic marker for survival interval. Survival time seems to be related to glial tumor type, tumor grade, percentage of cell staining, cell staining intensity, and specific pathologic tumor type ($P < 0.01$ for all). Among all gliomas, regardless of specific tumor pathology, median survival time was 70.6 months in patients whose tumors had no staining of CYP1B1. In contrast, median survival time was 19.6 months in patients whose tumors had strong staining (P = 0.0021). This finding is caused by the confounding influence of tumor grade on survival. Patients with higher-grade gliomas, which portend a shorter survival interval, were more likely to express the CYP1B1 antigen. The differences in survival time observed for patients in our study having different tumor grades and different specific pathologies conformed to expectations established for these tumor types. Patients with oligodendroglial tumors had a much longer median survival time than patients with astrocytomas (99.8 and 19.2 months, respectively; Table 4), the difference being attributed to the generally higher grade of astrocytomas relative to oligodendroglial tumors. As expected, survival interval decreased with increasing tumor grade in our study. Although percentage of tumor cells stained was related to survival time, the survival curves for <1% and 1% to 50% of cells stained overlay each other (Fig. 3). In contrast, patients with samples that had >50% of cells stained had a much lower survival rate. As with percentage of cells stained, tumor cell staining intensity was related to survival. The survival curves of patients having tumors showing no staining or low to moderate cell staining often overlapped (Fig. 4), but patients with tumors showing strong staining had a much shorter survival time. Similar to the results for cell staining percentage, this was also a function of tumor grade, with cell staining intensity increasing as did the grade of the tumor.

As established above, CYP1B1 expression correlated with the overall malignant behavior of the tumor, and thus it would be reasonable to expect it to function as a negative prognostic indicator within specific tumor pathologies as well. Yet, according to univariate analysis, within specific pathologic subtypes, cell staining intensity and the percentage of cell staining seemed to correlate positively with survival time. For example, among gliomas, such as glioblastomas, anaplastic astrocytomas, and oligodendrogliomas, there was a trend toward enhanced survival in patients that express CYP1B1 (Table 4), but this was not statistically significant. Any positive or negative correlation of CYP1B1 expression with median survival time in patients with low-grade gliomas and mixed oligoastrocytomas could not be determined, owing to the rarity of these tumors, insufficient patient deaths, and insufficient statistical power.

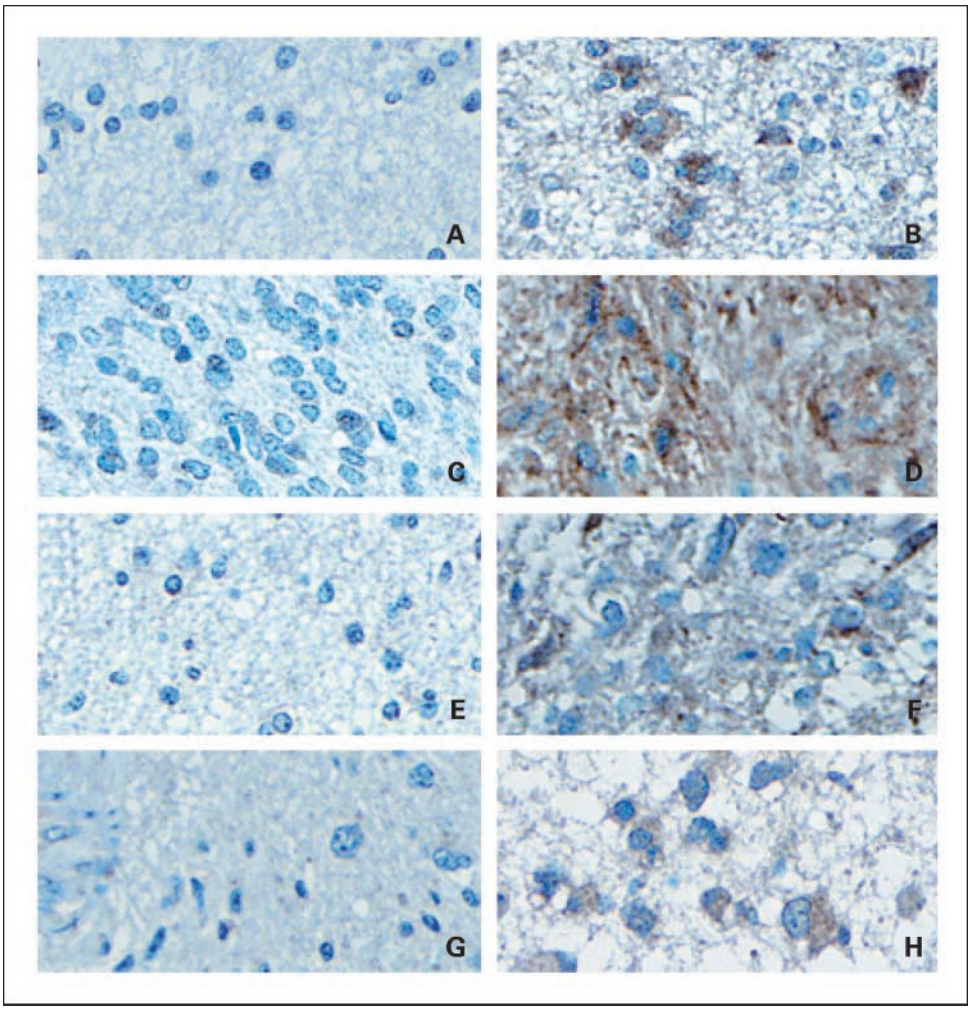

Fig. 2. Photomicrographs of staining results for oligodendrogliomas and mixed oligoastrocytomas. Magnification, ×200. *A,* oligodendroglioma showing negative staining for CYP1B1. *C,* anaplastic astrocytoma showing negative staining for CYP1B1. *E,* mixed oligoastrocytomas showing negative CYP1B1 staining. *G,* anaplastic mixed oligoastrocytoma showing negative CYP1B1 staining. *B,* oligodendroglioma showing positive staining for CYP1B1. *D,* anaplastic oligodendroglioma showing positive staining for CYP1B1. *F,* mixed oligoastrocytoma showing positive staining for CYP1B1. *H,* an anaplastic mixed oligoastrocytoma showing positive staining for CYP1B1.

However, when multivariate analysis was used to account for confounding factors, such as a patient's age and KPS score, no prognostic effect of CYP1B1 was found. When hazard ratios were computed for staining, KPS score, age, and specific pathology for the group as a whole, significant hazard ratios were found for age, KPS score, and all WHO III and IV tumors, establishing that the study sample conforms to established patterns of risk stratification. However, staining for CYP1B1 showed no tendency either toward increased or decreased survival time (data not shown). No effect was found on the hazard ratio when staining percentage and intensity were calculated separately versus combined. Hazard ratios were also computed for staining, age, and KPS score within each specific pathology, which again found no relationship between staining characteristics on survival.

Discussion

In this study, we have shown CYP1B1 expression throughout all major glioma subtypes and tumor grades. Furthermore, as tumors become more malignant, the amount and intensity of CYP1B1 expression increases. Within glial tumors, the CYP1B1 antigen is commonly expressed in glioblastoma, gliosarcoma, anaplastic astrocytoma, oligodendroglioma, anaplastic oligodendroglioma, mixed oligoastrocytoma, and anaplastic mixed oligoastrocytoma and often with significant staining intensity. For many of these glial tumors, such as oligodendrogliomas, significant immunotherapeutic targets have not been identified. Almost all immunotherapy clinical trials in gliomas have been for patients with anaplastic astrocytomas and glioblastoma multiforme. However, low-grade gliomas may represent the greatest potential for therapeutic approaches given the extended survival times afford sufficient time to generate immune responses. Thus, directing immunotherapy to CYP1B1 on these tumors provides a novel opportunity, especially because CYP1B1 can be recognized by tumor cytotoxic T cells.

As noted earlier, previous studies have shown that human T cells are capable of mounting an antigen-specific response to endogenously processed CYP1B1 peptide sequences (1) and that a vaccine targeting this antigen is capable of safely stimulating such a response *in vivo*, with some shown efficacy in various malignancies (5). Additionally, because vaccination against a single tumor antigen has been shown to produce negative revertants and subsequent loss of efficacy (12), the identification of viable immunologic targets, such as CYP1B1, is essential. However, because CYP1B1 expression is seen on cells of the fallopian tube, breast, and prostate, there is the theoretical potential of inducing autoimmunity. Although these tissues are not essential for patient survival, the inadvertent

induction of immune attack on the central nervous system, in contrast, could be more grave (17). Nevertheless, autoimmunity was not observed in patients treated with immunotherapy directed against the CYP1B1 antigen in the phase I clinical trial (5). Therefore, our study suggests that CYP1B1 represents a viable target for immunotherapy in malignant gliomas of both oligodendroglial and astrocytic lineages. Within the context of screening patient's tumors for expression of CYP1B1 as a

Table 4. Summary of analyses of survival of patients with glial tumors

		Median survival (mo)	Median KPS	Average age	No. patients	No. deaths	*P*
Glial tumor type	Astrocytoma	19.2	90	46.0	121	84	<0.0001
	Oligodendroglial	99.8	100	37.7	88	30	
GBM (WHO IV)	All	14.1	90	49.3	63	49	
	0 cells stained	12.7	90	44.4	12	9	0.8476
	1-50% cells stained	12.8	90	44.5	15	9	
	>50% cells stained	14.9	90	53.0	36	31	
	No stain	12.7	90	44.4	12	9	0.7173
	Light-moderate stain	12.8	90	51.2	31	26	
	Strong stain	18.1	90	49.4	20	14	
AA (WHO III)	All	27.7	90	42.7	43	28	
	0 cells stained	13.8	90	43.1	8	5	0.8284
	1-50% cells stained	28.1	90	39.6	24	16	
	>50% cells stained	27.7	95	49.2	11	7	
	No stain	13.8	90	43.1	8	5	0.0907
	Light-moderate stain	53.7	95	40.7	21	12	
	Strong stain	13.7	90	45.5	14	11	
O (WHO II)	All	99.8	100	38.5	37	11	
	0 cells stained	99.8	100	38.6	16	6	0.8528
	1-50% cells stained	NA	100	37.3	16	3	
	>50% cells stained	NA	100	41.8	5	2	
	No stain	99.8	100	38.6	16	6	0.0857
	Light-moderate stain	NA	100	37.2	17	3	
	Strong stain	NA	100	43.5	4	2	
AO (WHO III)	All	62.1	90	39.7	21	10	
	0 cells stained	NA	95	43.6	8	2	0.0541
	1-50% cells stained	62.1	100	35.7	7	3	
	>50% cells stained	9.3	90	39.2	6	5	
	No stain	NA	95	43.6	8	2	0.2747
	Light-moderate stain	14.0	90	42.7	9	5	
	Strong stain	42.8	95	25.3	4	3	
AMOA (WHO III)	All	89.2	95	35.2	18	8	
	0 cells stained	23.2	95	36.9	8	5	0.0279*
	1-50% cells stained	NA	90	33.2	6	0	
	>50% cells stained	59.0	100	35.0	4	3	
	No stain	23.2	95	36.9	8	5	0.0543
	Light-moderate stain	NA	90	33.7	7	1	
	Strong stain	89.2	100	34.3	3	2	
GS	All	5.4	80	51.0	8	6	
	0 cells stained	18.7	75	58.7	3	2	0.1015
	1-50% cells stained	4.1	75	49.7	3	2	
	>50% cells stained	4.9	90	41.5	2	2	
	No stain	18.7	75	58.7	3	2	0.0214
	Light-moderate stain	2.9	90	51.0	1	1	
	Strong stain	5.3	80	45.3	4	3	
LGA	All	NA	95	29.7	7	1	
	0 cells stained	NA	90	27.7	3	1	0.6065*
	1-50% cells stained	NA	100	29.3	3	0	
	>50% cells stained	NA	NA	37.0	1	0	
	No stain	NA	90	27.7	3	1	0.3173*
	Light-moderate stain	NA	100	33.0	3	0	
	Strong stain	NA	100	26.0	1	0	
MOA	All	NA	100	35.4	12	1	
	0 cells stained	NA	100	33.9	7	1	0.6997*
	1-50% cells stained	NA	95	39.3	4	0	
	>50% cells stained	NA	70	31.0	1	0	
	No stain	NA	100	33.9	7	1	0.6997*
	Light-moderate stain	NA	80	35.8	4	0	
	Strong stain	NA	100	45.0	1	0	

*P value questionable because some strata have no events.

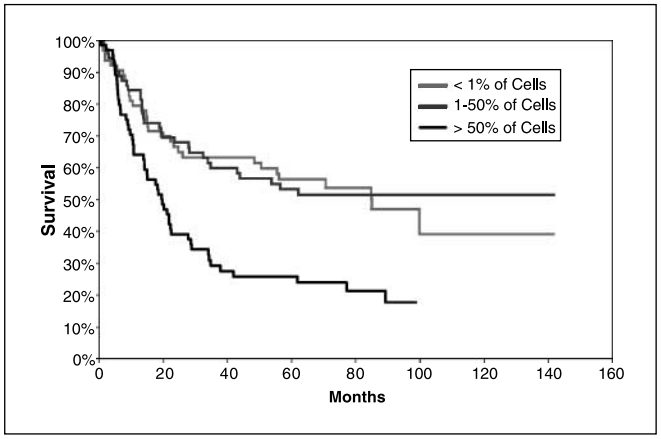

Fig. 3. Kaplan-Meier curves showing the median survival in all patients with a glioma by percentage of cell staining. Patients (n = 65) with <1% staining had a median survival of 84.9 mon, those (n = 77) with 1% to 50% had an inconclusive median survival secondary to insufficient deaths, and in those (n = 66) with more 50% staining the median survival was 19.6 mon (P < 0.0001).

criterion for targeted therapy, the percentage of cell staining would be most pertinent because this perimeter is more reliable across assays. Immunohistochemical staining for intensity is only semiquantitative, and even moderate differences in intensity staining could be relatively meaningless if there is not normalization across all specimens as we have done within this study.

CYP1B1 expression correlated positively with tumor grade and paradoxically seemed to have a positive effect on survival within specific diagnoses on univariate analysis; however, in a multivariate analysis, it was not shown to affect survival either positively or negatively. Although CYP1B1 increased expression of CYP1B1 has been shown in breast, colorectal, and other cancers, it has not been found to be a prognostic marker for survival in these types of cancers either (15, 18). In the case of colorectal cancers, the cytochrome *P*450 CYP51 was found to be an independent marker of prognosis, and in ovarian cancers, the cytochrome *P*450s CYP2A/2B and CYP4Z1 were markers of poor prognosis. Cumulatively, these data indicate that there is tumor-specific expression of individual cytochrome *P*450s. This may be related to the hypoxic environment of the tumor or its metastatic potential. For example, the expression of CYP51 may promote tumor motility, invasive capacity, and subsequent metastasis (15). In gliomas, which rarely metastasize outside the central nervous system and are within a hypoxic environment, the expression of CYP1B1 may be more critical. Other antigenic prognostic markers have been identified within gliomas. The epidermal growth factor variant III is a tumor-specific antigen that has served as a successful target for immunotherapies (10, 11), but its role as a negative prognostic marker is controversial (14, 19). Tenascin-C has also been shown to be a negative prognosticator, but it is only expressed in the extracellular matrix rather than on the tumor cell surface (20). Expression of survivin has also been correlated with pathologic grade of the tumor (21), and its enhanced expression within glioblastoma patients is a negative prognosticator (21).

CYP1B1 may play a role in the tumorigenesis of gliomas. It has been shown that CYP1B1 is a metabolic activator of polycyclic aromatic hydrocarbons, such as those found in cigarette smoke. CYP1B1 can convert polycyclic hydrocarbons to diols and epoxides, which interact with DNA, forming stable adducts and single-strand breaks, and have been implicated in the induction of mutations in the *p53* gene (22). This is consistent with previous work showing that p53 mutations are prevalent in glioblastomas (23) and oligodendrogliomas (24, 25). Previous studies have also shown that p53 mutations and chromosome 1p/19q loss are mutually exclusive within both astrocytomas and oligodendrogliomas (26). Notably, the mechanism of p53 mutation is consistent with the molecular action of CYP1B1, whereas that of loss of heterozygosity at chromosomes 1p and 19q is not. This supports our finding of low levels of CYP1B1 staining in oligodendrogliomas exhibiting deletions of 1p/19q despite the small number of patients. In a study that seems to contradict this hypothesis, which examined the risk of adult brain tumors as a function of CYP1B1 gene polymorphism, no correlation was found between genetic polymorphisms at the CYP1B1 gene locus and increased risk of glial tumors (27); however, in the authors' primary analysis, low-grade glial tumors were included with high-grade gliomas, and no adjustment was made for the difference in levels of expression of the CYP1B1 enzyme. As we have shown in this study, there is a significant difference in the level of CYP1B1 expression between these grades of tumor. Therefore, it is possible that one polymorphic variant is found in greater numbers in patients whose tumors express CYP1B1 or whose tumors tend to progress beyond the lower grades, leaving the effect of genetic polymorphism on glioma risk still unresolved.

The overexpression of CYP1B1 in gliomas may be rendering them either chemoresistant or radioresistant. Overexpression of CYP1B1 has been shown to increase the occurrence of ssDNA breaks (28), and the cytochrome *P*450s are involved in the oxidative metabolism (both activation and deactivation) of many anticancer drugs. The cytochrome *P*450s are capable of influencing the responses of tumors to a variety of anticancer therapies (29, 30). Additionally, radioresistant cancer cell lines have been shown to overexpress CYP1B1 on cDNA microarrays (31). Finally, in patients vaccinated against CYP1B1 who then received salvage chemotherapy, there were significant clinical

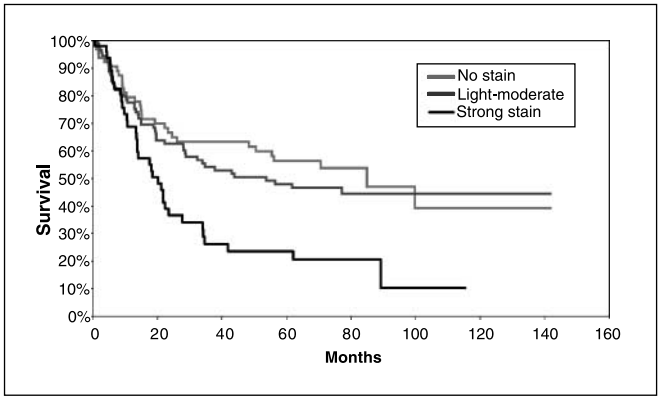

Fig. 4. Kaplan-Meier curve showing the median survival in all patients with a glioma by intensity of cell staining. Patients (n = 65) with no staining had a median survival of 84.9 months, those (n = 92) with light to moderate staining had a median survival of 53.7 mon, and in those (n = 51) with strong staining the median survival was 20.1 mon (P = 0.0014).

responses (5). One could hypothesize that the elimination of CYP1B1 expression could contribute to the survival advantage.

In conclusion, CYP1B1 seems to be overexpressed in glial cell tumors and represents a viable target for immunotherapy with agents such as ZYC300. Whether it functions as a procarcinogen in the tumorigenesis of gliomas or as a chemosensitizer in their treatment is an issue inviting further study. It may in fact do both, with results that are entirely substrate dependent.

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