Dysregulation of PTEN and Protein Kinase B Is Associated with Glioma Histology and Patient Survival

Ralph P. Ermoian, Constance S. Furniss, Kathleen R. Lamborn, Daniel Basila, Mitchel S. Berger, Alexander R. Gottschalk, M. Kelly Nicholas, David Stokoe, and Daphne A. Haas-Kogan


ABSTRACT

Purpose: This study assessed whether putative effectors of phosphatidylinositol 3-kinase, including PTEN, protein kinase B (PKB), and p27kip1, correlate with each other, with glioma histology, and with patient outcome.

Experimental Design: Components of the phosphatidylinositol 3-kinase signaling cascade were characterized in 25 glioblastoma multiforme (GBM) tumors, 8 grade II oligoastrocytomas, and 13 normal human brain specimens. The protein levels of PTEN and p27kip1 were assessed by immunoblot analyses. PKB kinase activity was evaluated through the expression level of the phosphorylated (activated) PKB protein and the ability of PKB to phosphorylate a specific peptide substrate in vitro. Cox regression analyses between expression/activity variables and survival were performed across and within histology types. Actual value for expression/activity was used as a continuous variable. Survival time was displayed by Kaplan Meier.

Results: A strong inverse correlation was evident between PTEN levels and both phosphorylated PKB expression (P < 0.01) and PKB activity (P = 0.01). p27kip1 levels did not correlate with PTEN expression or PKB activity. A significant association was evident between PTEN expression level and histology (P < 0.01). PTEN levels were highest in normal brain, lowest in GBM tumors, and intermediate in grade II oligoastrocytomas. PKB activity and phosphorylated PKB levels differed significantly among histologies, whereas p27kip1 levels exhibited no association with histology. PTEN expression correlated significantly with survival time within the entire cohort (P < 0.01) and was associated with survival within the subgroup of GBM tumors (P = 0.11).

Conclusions: Reduced PTEN expression is ubiquitous among GBM tumors and may play a role in the development of low-grade gliomas. PTEN inactivation in gliomas portends a particularly aggressive clinical behavior.

INTRODUCTION

Gliomas are the most common primary intracranial tumor. Although tumor grade predicts the clinical course of most patients, molecular characteristics of individual tumors have emerged as important prognostic factors for patients with glioma brain tumors (1). Such molecular tumor characteristics can help define prognosis for individual patients. Molecular analysis of GBM is of particular interest because no curative therapy is available currently for this neoplasm, and as a result, GBM tumors are uniformly fatal. Molecular profiling of GBM tumors may thus define the critical genetic alterations that underlie glioma pathogenesis and their marked resistance to therapy. Furthermore, elucidation of these critical molecular events will identify the most suitable pathways to target with novel therapeutic agents.

Multiple genetic pathways are altered in gliomas, including p16/pRb/CDK4, p53/MDM2/p14ARF, epidermal growth factor receptor, platelet-derived growth factor receptor, and PI3-kinase/PTEN. Few alterations, however, predict consistently the clinical outcome (1). The most common genetic alteration in GBM tumors is loss of heterozygosity of chromosome 10, occurring in 80 to >95% of these tumors (2, 3). PTEN is a putative tumor suppressor gene cloned from 10q23.3; however, only 20–44% of primary GBM tumors harbor mutations in PTEN (4–8). Several recent studies have reported that mutations in the PTEN (MMAC1/TEP1) tumor suppressor are a significant predictor of poor survival among patients with high-grade gliomas. This implicates PTEN, and the pathway it mediates, as an important determinant of glioma aggressiveness and a key target for therapeutic intervention.

PTEN regulates several cellular functions, including migration, invasion, survival, proliferation, and angiogenesis, by antagonizing PI3-kinase-mediated signaling cascades (9). PTEN dephosphorylates the 3’ phosphate on lipid second-messenger products of PI3-kinase, and mutations of PTEN lead to constitutive activation of this critical signaling pathway. Reintroduc-
tion of wild-type PTEN into glioma cells harboring mutant PTEN induces an arrest in the G1 phase of the cell cycle (10–13). Downstream effectors that mediate the PTEN-induced growth arrest have been identified in vitro, including the serine/threonine kinase PKB/Akt (14) and the cyclin-dependent kinase inhibitor p27kip1 (11, 15). Although these downstream molecules have been established in vitro, in vivo studies have yet to establish which of these PI3-kinase/PTEN-effectors is important for glioma pathogenesis. To this end, we examined primary human gliomas of different grades, as well as normal brain specimens, and correlated the expression level and activity of putative downstream effector proteins of PI3-kinase. Correlations were further sought between these putative effectors and patient survival.

Signaling molecules in the PI3-kinase cascade have been shown previously to predict prognosis and survival of patients with various cancers. Decreased p27kip1 expression is a strong predictor of poor clinical outcome in several human tumors, including malignant melanomas, non-Hodgkin’s lymphomas, as well as non-small cell lung, colon, breast, prostate, gastric, laryngeal, and ovarian carcinomas (16). However, little is known about the prognostic significance of p27kip1 expression in gliomas. There are conflicting reports regarding the prognostic significance of PTEN in human malignancies. An association with poor survival, a correlation with improved prognosis, and no significance of PTEN in human malignancies. An association with poor survival, a correlation with improved prognosis, and no significance of PTEN in human malignancies.

MATERIALS AND METHODS

Patients and Tissue Samples. Tissues from 46 patients who were treated at University of California, San Francisco between 1990 and 1999 were analyzed. Our goal was to acquire 30 GBM samples to generate a statistically meaningful cohort. Of these 30 samples, 5 contained ≤70% tumor upon histological examination and therefore were not evaluated. The number of normal brain and OA samples was dictated by the maximal number of each that was available during the study acquisition period. A summary of relevant patient data is displayed in Table 1. Tissues were frozen immediately after surgery and stored at −80°C at the University of California, San Francisco Brain Tumor Research Center until needed for molecular analysis. Appropriate institutional board approval was acquired. Formalin-fixed, paraffin-embedded sections were prepared for all tissues and reviewed by a neuropathologist. GBM and OA samples consisting of >70% tumor were chosen for further study. Normal brain specimens were acquired from 13 patients undergoing surgery for epilepsy and were reviewed to verify the absence of tumor.

Tissue Homogenization. Frozen tissue specimens were thawed and homogenized. A portion measuring 100–300 mg of each tissue sample was manually homogenized in a Dounce homogenizer ( Kontes ) in 0.5–1 ml of 10% NP40 lysis buffer and incubated for 10 min at 0°C. After centrifugation at 4°C, the supernatant was further homogenized by sonication using a Sonic Dismembrator (Fisher Scientific).

Immunoblot Analysis. Protein concentrations for each lysate were quantified using the Lowry assay (Bio-Rad). Expression levels of PTEN, p27kip1, and phosphorylated (active) PKB were determined for all OA, GBM, and normal brain samples by immunoblot analysis. Expression of these proteins was normalized to β-actin levels. Cell lysates were diluted 1:1 in SDS-PAGE loading buffer. Twenty μg of protein per tissue lysate were electrophoresed on a 10% Tris/glycine SDS-polyacrylamide gel and transferred to Immobilon P membranes (Millipore). The membrane was blocked with 5% nonfat dry milk in TBST (Tris-buffered saline containing 0.1% Tween 20). The membrane was then incubated with the primary antibody in 5% nonfat dry milk in TBST, followed by a secondary antibody linked to horseradish peroxidase diluted in 5% nonfat dry milk in TBST. The membranes were chemically stripped of antibodies using stripping buffer [ 2% SDS, 20 mM Tris (pH 6.5), and 7 mM β-mercaptoethanol] and reblocked with nonfat milk in 1 × TBST before application of successive antibodies. The ECL Detection System for Western blot analysis (Amersham) was followed according to the manufacturer’s instructions for antibody detection. AlphaImager 2000 Documentation and Analysis System (Multi Image light cabinet photodensitometer) was used to quantify bands of appropriate sizes ( Alpha Innotech Corp.). The following primary antibodies were used: anti-β-actin ( Sigma Chemical Co.), anti-PTEN (Cascade BioScience), anti-p27kip1 (Transduction Laboratories), and anti-phosphorylated PKB (20).

PKB Kinase Activity Assay. Equal amounts of total protein were subjected to immunoprecipitation with antibodies generated against recombinant full-length PKB. Immunoprecipitates were washed twice and assayed for PKB activity using 50 μM Crosstide (GRPRSSFAEG) in a volume of 10 μl. The assay was terminated by adding an equal amount of 2× Tricine sample buffer, and the phosphorylated peptide was separated from free [ γ 32 P]ATP by electrophoresis on 16% Tricine gels. Phosphorylation of Crosstide was quantitated using a Storm phosphorimager ( Molecular Dynamics).

<table>
<thead>
<tr>
<th>Table 1</th>
<th>Patient characteristics</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Normal brain (n = 13)</td>
</tr>
<tr>
<td>Sex (female: male)</td>
<td>6:7</td>
</tr>
<tr>
<td>Median age (range) in years</td>
<td>35 (14–52)</td>
</tr>
<tr>
<td>Median survival (95% CI) in months</td>
<td>Not applicable</td>
</tr>
<tr>
<td>No. deceased</td>
<td>0</td>
</tr>
</tbody>
</table>

* CI, confidence interval.
Statistical Analysis. SPSS for Windows, Version 10.0.07 (SPSS, Inc., Chicago, IL) was used in statistical analyses and preparation of figures. All Ps reported are from two-tailed hypothesis tests. Association between PTEN expression, PKB activity, phospho-PKB expression, and p27kip1 expression levels were assessed using Spearman’s ρ correlation. Kruskal-Wallis tests were performed on the expression/activity levels of the variables between samples of different histologies, and box plots were generated. To address the multiple comparisons issue, an overall analysis was done comparing the three groups. Only if this analysis was statistically significant were individual pairwise comparisons done. Finally, Cox regression analyses between the expression/activity variables listed previously and survival were performed both across and within histology types. The actual value for expression/activity was used as a continuous variable for these analyses. For the assessment of expression/activity as a predictor among GBM cases, analyses were done with and without adjustment for age. For display purposes, expression/activity was graded as high/low based on the median level of that variable, and survival time was displayed using the method of Kaplan Meier. Deaths were verified by examination of medical records and the Social Security Death Index. In one patient with grade II OA, insufficient survival data were available to include that patient in survival analyses. In cases where no death could be confirmed, survival was assumed to be the date of the last clinic visit or correspondence with a physician.

RESULTS

Characterization of PTEN, p27kip1, and PKB in Primary Gliomas. In vitro biochemical studies have indicated that PTEN, PKB, and p27kip1 function within the PI3-kinase signaling pathway. To explore whether these molecules are important mediators of PI3-kinase activity in vivo, we characterized the status of PTEN, PKB, and p27kip1 in primary human specimens of GBM tumors, grade II OAs, and normal brain tissue. To identify tumors with decreased PTEN function, we assessed PTEN protein expression levels. We reasoned that decreased PTEN expression would identify tumors with gene mutations, because some mutations have been reported to reduce PTEN stability and expression (21) and would additionally identify tumors in which PTEN expression is silenced because of methylation or promoter mutations. However, point mutations that do not cause decreased stability would be missed in this analysis (see “Discussion”). Our assessment of p27kip1 also focused on protein levels because p27kip1 expression carries significant prognostic value in a myriad of human malignancies (22). In characterizing the status of PKB, a known downstream target of PI3-kinase, we assayed its kinase activity, reflected by both the expression level of the phosphorylated (activated) PKB protein (23) as well as the ability of PKB to phosphorylate a specific peptide substrate in vitro (24).

We characterized PTEN, p27kip1, and PKB, as well as β-actin as a loading control, in 25 GBM tumors, 8 grade II OAs, and 13 specimens of normal human brain. On the basis of the PI3-kinase pathway model described previously (9), we anticipated that PTEN expression would correlate directly with p27kip1 levels but correlate inversely with PKB activity. Furthermore, we predicted that a subset of the GBM tissue samples would demonstrate decreased PTEN expression, resulting in increased phosphorylated PKB expression, increased PKB activity, and decreased p27kip1 expression. Because PTEN mutations are virtually absent from grade II gliomas, we hypothesized that the grade II OA and normal brain specimens would display identical patterns of PTEN, p27kip1, and PKB.

The actual value for expression/activity was used as a continuous variable for these analyses. For the assessment of expression/activity as a predictor among GBM cases, analyses were done with and without adjustment for age. For display purposes, expression/activity was graded as high/low based on the median level of that variable, and survival time was displayed using the method of Kaplan Meier. Deaths were verified by examination of medical records and the Social Security Death Index. In one patient with grade II OA, insufficient survival data were available to include that patient in survival analyses. In cases where no death could be confirmed, survival was assumed to be the date of the last clinic visit or correspondence with a physician.

PTEN Levels and PKB Activity Strongly Correlate with Tumor Grade. We sought to determine whether PTEN, phosphorylated PKB, and p27kip1 expression levels, as well as PKB activity, correlate with tumor grade. The scatter plots in Fig. 2, A and B, reveal that normal brain samples cluster in a region of...
the graph representing high PTEN levels, GBM samples cluster in an area depicting low PTEN levels, and grade II OAs cluster between the normal brain and GBM groups. To further analyze an association between PTEN expression level and tumor grade, the data were displayed in box plots by histology (normal brain, grade II OA, and grade IV glioblastoma multiforme) tissue samples. Values are from a single experiment representative of three independent experiments. A Spearman’s correlation coefficient (SCC) and $P$ are shown for each plot.

Fig. 2  PTEN expression plotted against phosphorylated PKB levels (A), PKB kinase activity (B), or p27$^{kip1}$ levels (C) as well as phosphorylated PKB levels (D) plotted against p27$^{kip1}$ expression in normal (NL) brain (○), grade II OA (●), and grade IV glioblastoma multiforme (□) tissue samples. Values are from a single experiment representative of three independent experiments. A Spearman’s correlation coefficient (SCC) and $P$ are shown for each plot.

Fig. 3  Boxplots depicting PTEN levels (A), PKB kinase activity (B), phosphorylated PKB levels (C), and p27$^{kip1}$ levels (D) in normal (NL) brain, grade II OA, and grade IV GBM tissue samples. Black horizontal line, the median value for each group; gray box, the middle 50% of the values, lying between the 25 and 75%; horizontal gray lines, represent the minimum and maximum values observed in each group. For example, the graph demonstrates that the maximum PTEN level among GBM tumors is slightly lower than the minimum PTEN level among normal brain samples. Statistical analysis was performed using the Kruskal-Wallis test.
diate in grade II OAs. The boxplots demonstrate that the maximum PTEN level among GBM tumors is slightly lower than the minimum PTEN level among normal brain samples. Furthermore, pairwise comparisons of grade II OA versus normal brain, GBM versus normal brain, and grade II OA versus GBM all demonstrated statistically significant differences in PTEN expression levels (P < 0.01 for all pairwise comparisons).

This analysis was repeated for PKB activity, phosphorylated PKB levels, and p27kip1 expression. PKB activity and phosphorylated PKB levels differed significantly among histologies (P = 0.02 in Fig. 3B and P < 0.01 in Fig. 3C). Further pairwise analyses revealed that PKB activity and phosphorylated PKB levels differed significantly between grade II OA and normal brain (P = 0.04 and P < 0.01, respectively) as well as between GBM and normal brain (P < 0.01 for both PKB activity and phosphorylated PKB levels) but not between grade II OA and GBM specimens (P = 0.24 and P = 0.53, respectively). p27kip1 levels exhibited no association with histology (P = 0.87; Fig. 3D).

**PTEN Expression Is a Predictor of Patient Survival Time.** PI3-kinase is a multifactorial signaling molecule with a number of downstream targets that participate in diverse cellular functions. It remains unclear which of these functions plays a key role in glioma pathogenesis. To shed light on this question, we sought to determine which components of the PI3-kinase pathway are predictors of survival in glioma patients. Of 8 patients with grade II OA, 1 was lost to follow up, 1 died 332 weeks after diagnosis, and median follow-up for the living patients was 199 weeks (range, 10–411 weeks). Of 25 GBM patients, 24 died of their disease and 1 patient was followed for 29 weeks and was then lost to follow up (Table 1).

Kaplan-Meier curves and Cox regression analyses of all tumors, including both grade II OAs and GBMs, demonstrated a statistically significant positive correlation between patient overall survival time and PTEN expression level (P < 0.01; Fig. 4A). This result is consistent with the inherent worse survival of GBM patients and the association we found between tumor grade and PTEN levels. To extend this observation, we reviewed grade II OA tumors and GBM tumors separately and asked whether within each subgroup PTEN expression predicts patient survival. The association between PTEN and survival could not be addressed within the grade II OA cohort because only 1 death has occurred among these patients. When patients with GBM tumors were analyzed separately, high PTEN expression still appeared to be prognostic, although with the small sample size the results were not significant statistically (Fig. 4B; P = 0.11, Cox proportional hazards, estimated hazard ratio 0.2/unit increase in PTEN). Adjustment for age did not alter these results. Similar Kaplan-Meier curves and Cox proportional hazards regression analyses revealed no indication of a correlation between patient survival time and either p27kip1 expression, phosphorylated PKB levels, or PKB activity for the subgroup of GBM patients (P = 0.84, Fig. 4C; 0.52, Fig. 4D; and 0.46, data not shown, respectively).
DISCUSSION

The PI3-kinase signaling pathway is constitutively activated in a significant proportion of GBM tumors. Activation of this pathway likely contributes to the development of GBM tumors and to their exquisite resistance to standard therapies (9). Although in vitro biochemical studies have identified putative components of the PI3-kinase pathway, it remains unclear which of these elements plays a salient role in glioma development in vivo. Identification of these key elements is important because they likely represent the most promising molecular targets for signal transduction inhibitors.

We chose to characterize PTEN, p27^{kip1}, and PKB from the molecules known to impinge on the PI3-kinase pathway in vitro. Our choice of PTEN stemmed from several recent publications in which PTEN inactivation is a poor prognostic factor in high-grade gliomas as well as in several other human malignancies. PTEN inactivation in gliomas has been documented by the presence of mutations in the PTEN coding region (25, 26), decreased mRNA levels as measured by reverse-transcription PCR (27), and reduced protein expression as assessed by immunohistochemistry (27). PTEN inactivation by any of these mechanisms has been reported to portend poor clinical outcome in malignant gliomas. In this study, PTEN was assessed by immunoblot analysis to quantitate protein expression levels. We reasoned that decreased PTEN expression would identify tumors with gene mutations, because some mutations have been reported to reduce PTEN stability and expression (21) and would additionally identify tumors in which PTEN expression is silenced because of methylation or promoter mutations. Although it is expected that this approach would miss PTEN mutations that do not affect protein stability, our data demonstrate that every GBM tumor exhibited lower PTEN expression than each of 13 normal brain specimens, indicating that analysis of PTEN expression level likely detected the vast majority of tumors with PTEN inactivation.

The cyclin-dependent kinase inhibitor p27^{kip1} was chosen for this study because the expression level of p27^{kip1} carries significant prognostic value in a myriad of human malignancies (22), and p27^{kip1} mediates the growth-suppressive function of PTEN in vitro (9, 11–15). PKB was evaluated because, as a direct phosphorylation target of PI3-kinase, the state of activation of PKB reflects endogenous PI3-kinase activity (9). To identify the key components of the PI3-kinase pathway that will prove the most valuable targets for therapeutic inhibition, we sought to identify the signaling molecules that exhibited the strongest correlation with: (a) PTEN inactivation; (b) tumor grade; and (c) patient survival. PTEN inactivation, as reflected by reduced PTEN expression, correlated with PKB kinase activity, as measured by both the expression level of phosphorylated (activated) PKB protein (23) and the ability of PKB to phosphorylate a specific peptide substrate in vitro (24). These results are similar to those reported by the sole previous study that examined PKB activity in primary human tumors. In this study of primary epithelial ovarian carcinomas, phosphorylated PKB levels showed significant association with PTEN status in vivo. In vitro PKB kinase activity was not assessed in this report (28).

Interestingly, the p27^{kip1} expression level did not correlate with PTEN expression, phosphorylated PKB level, or PKB kinase activity. These results were unexpected, because previous studies had indicated that the PI3-kinase pathway can regulate the p27^{kip1} level (9): (a) expression of PTEN in cultured glioma cells results in increased p27^{kip1} levels (12, 13); (b) inhibition of p27^{kip1} expression using antisense oligonucleotides impedes the PTEN-induced growth arrest (11, 15); and (c) p27^{kip1} is a direct transcriptional target of members of the Forkhead family (14), transcription factors that are controlled by PKB phosphorylation (29, 30). Our data are again consistent with the recent study of ovarian carcinomas, in which PTEN was assessed for intragenic mutations and immunohistochemical evidence of reduced protein expression, and was then tested for associations with phosphorylated PKB, p27^{kip1}, and cyclin D1. Although PKB, p27^{kip1}, and cyclin D1 have all been linked biochemically to the PI3-kinase pathway, only phosphorylated PKB levels showed significant association with PTEN status in ovarian cancer in vivo. Our results indicate that the regulation of p27^{kip1} in human gliomas is multifactorial and does not correlate directly with PI3-kinase pathway activity.

PTEN, p27^{kip1}, and PKB were assayed not only for associations with each other, but also for correlation with tumor grade. A molecular feature that strongly associates with the markedly aggressive GBM histology, as compared with lower grade gliomas and normal brain, is more likely to contribute to the biological and clinical behavior of GBM tumors. Although p27^{kip1} levels exhibited no association with tumor grade, PTEN inactivation and PKB activity showed significant association with tumor grade. Specifically, PTEN expression was lowest in GBM tumors, a finding consistent with previous reports in which PTEN mutations are commonly found in high-grade tumors (31). Furthermore, a key finding of our study is that grade II OA tumors exhibited an intermediate level of PTEN expression, lower than normal brain, but higher than GBM tumors. Although PTEN mutations are virtually never found in grade II gliomas, previous studies have focused on mutations in the coding region of PTEN, whereas the current report focuses on PTEN protein expression levels. The only other study that evaluated PTEN expression level, assayed by semiquantitative reverse transcription-PCR, examined gliomas of all grades and reported that relative PTEN expression was associated with patient survival time if no adjustment was made for histological grade (27). Our data, in conjunction with this report, may implicate PI3-kinase dysregulation in the development of lower grade gliomas, a novel finding that requires further study.

Finally, components of the PI3-kinase pathway were analyzed for an association with patient survival. From these analyses, a pattern emerges linking PTEN aberrations with aggressive clinical behavior of gliomas:

(a) When all glioma patients were examined as a whole, reduced PTEN expression level was a predictor of poor survival.

(b) PTEN expression in grade II OA tumors held an intermediate level between normal brain and GBM tumors, consistent with their intermediate clinical phenotype, which is clearly malignant compared with normal brain tissue but less aggressive when compared with GBM tumors.

(c) Within the subgroup of GBM tumors, the data suggest a potential association between lower PTEN levels and shorter patient survival. Taken together, our results implicate PTEN aberrations and PI3-kinase dysregulation in promoting aggres-
Repressive biological behavior of gliomas, a finding consistent with several previous reports in which PTEN inactivation correlates with poor clinical outcome (25–27).

This study supports an enlarging body of literature that demonstrates that PTEN inactivation in gliomas portends a particularly aggressive clinical behavior. The PI3-kinase pathway may thus represent a promising molecular target for therapeutic intervention. These findings add to the enthusiasm of targeting PI3-kinase in glioma therapy, because of the prevalent nature of PTEN aberrations in GBM tumors, the association between PTEN aberrations and aggressive clinical behavior, and the potential role of PI3-kinase dysregulation in lower grade gliomas. Novel signaling inhibitors have already entered clinical trials for gliomas, and we eagerly await their results.

ACKNOWLEDGMENTS

We thank the University of California, San Francisco Brain Tumor Research Center Tissue Bank for providing and sectioning all tissue samples.

REFERENCES


Dysregulation of PTEN and Protein Kinase B Is Associated with Glioma Histology and Patient Survival

Ralph P. Ermoian, Constance S. Furniss, Kathleen R. Lamborn, et al.


Updated version  Access the most recent version of this article at:
http://clincancerres.aacrjournals.org/content/8/5/1100

Cited articles  This article cites 31 articles, 14 of which you can access for free at:
http://clincancerres.aacrjournals.org/content/8/5/1100.full.html#ref-list-1

Citing articles  This article has been cited by 46 HighWire-hosted articles. Access the articles at:
/content/8/5/1100.full.html#related-urls

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.