The Prognostic Significance of Phosphatidylinositol 3-Kinase Pathway Activation in Human Gliomas

Arnab Chakravarti, Gary Zhai, Yoshiyuki Suzuki, Sormeh Sarkesh, Peter M. Black, Alona Muzikansky, and Jay S. Loeffler

ABSTRACT

Purpose
The objectives of this study were to examine activation patterns of the phosphatidylinositol 3-kinase (PI3K) pathway in gliomas and to examine the prognostic significance of PI3K pathway activation using snap-frozen clinical specimens.

Materials and Methods
Levels of expression of PI3K pathway members were assessed in 92 prospectively collected gliomas through quantitative Western analysis using total and phospho-specific antibodies for PI3K, Akt, and p70s6k. Both expression and expression levels of these PI3K pathway members were correlated with histology, markers of apoptosis (cleaved caspase 3), and with clinical outcome (eg, overall survival).

Results
It was determined that activation of all three PI3K pathway members were significantly more frequent in glioblastoma multiforme than in non-glioblastoma multiforme tumors. Levels of phospho-PI3K, phospho-Akt, and phospho-p70s6k were all found to be inversely associated with cleaved caspase 3 levels, suggesting PI3K pathway activation is associated with reduced levels of apoptosis. Perhaps most importantly, activation of PI3K pathway members was found to be significantly associated with reduced survival times when all glioma cases were considered in aggregate. When glioblastoma cases were considered separately, the prognostic value of PI3K activation remained significant, suggesting that PI3K activation may directly be associated with radiation resistance, given that this was the only adjuvant therapy administered to this subset of patients.

Conclusion
Activation of the PI3K pathway is significantly associated with increasing tumor grade, decreased levels of apoptosis, and with adverse clinical outcome in human gliomas. Molecular pathways regulating PI3K activation would appear to be promising targets in the clinical management of glioma patients.

INTRODUCTION

Gliomas are among the most treatment-refractory of all human tumors. The most malignant subtype, glioblastoma multiforme (GBM), is particularly aggressive, with median survivals under 1 year in most series, despite aggressive surgery, radiation, and chemotherapy.1-5 Recently, there has been much interest in identifying molecular and genetic pathways that are not only involved in gliomagenesis, but which may also be involved in mediating resistance to conventional therapies, such as chemotherapy and radiation.6-7 As one prime example, there has been much investigation into the prognostic and, potential therapeutic value of, signaling through the epidermal growth factor receptor (EGFR) pathway.8-14 Signal transduction pathways downstream of EGFR have been found to be critical in mediating important cellular functions, including survival, proliferation, migration, and invasion.15-23 These pathways include phosphatidylinositol 3-kinase (PI3K), RAS-RAF-mitogen-activated protein kinase, and protein kinase C, among others.

The PI3K pathway, in particular, appears to be one of the most potent pro-survival signaling pathways. Critical PI3K
mediators, including Akt and p70S6K, are known to actively suppress apoptosis and enhance cellular survival. While activation patterns of PI3K family members have been studied in preclinical models, there is a relative paucity of data regarding activation patterns of PI3K family members in clinical specimens and their potential clinical prognostic values. Further, the role of PI3K signaling in mediating radiation resistance in GBMs has been suggested in preclinical models, but has yet to be studied clinically. With the advent of phospho-specific antibodies, it is now possible to evaluate the activation states of critical signaling pathways in human tissues. Certainly, such information on the proteomics level is complementary to that obtained with gene expression profiling alone, and has the potential to be of important prognostic and therapeutic significance. In our study, we investigated these issues in 92 prospectively collected snap-frozen glioma specimens using quantitative Western analysis with phospho-specific antibodies directed against PI3K, Akt, and p70S6K.

**MATERIALS AND METHODS**

*Tissue processing.* Ninety-two glioma specimens have been prospectively collected (at Brigham and Women’s Hospital [BWH], Boston, MA) under an institutional review board approved protocol for the purpose of studying treatment resistance markers in gliomas. The histopathologic breakdown of these tumors is shown in Table 1. Each specimen was snap-frozen after surgical removal, carefully sectioned and histopathologically reviewed. Tissue containing greater than 95% tumor was provided for this analysis. Each tumor tissue was homogenized in either RIPA (lysis) buffer (for isolation of phospho-activated and total PI3K/Akt/p70S6K) or Chaps buffer (for isolation of cleaved caspase 3) using a Polytron homogenizer (Fisher, Pittsburgh, PA) to generate protein lysates for Western blot analysis.

*Patient data.* Histopathologic diagnosis was provided by BWH pathologists. Clinical data was obtained from the BWH tumor registry and from hospital charts. This data included patient age at diagnosis, treatment type, treatment dates, and clinical/survival outcomes. Patients with GBM and anaplastic astrocytoma were generally managed by surgery alone, with radiation deferred for progressive disease. The mean age of patients at the time of diagnosis was 48 years. The total number of deaths was 60 patients, with 32 patients censored. The median follow-up for patients that were alive at last follow-up was 16.5 months. Westerns were performed in a blinded manner, without prior knowledge of clinical outcome. Patient follow-ups were updated at the time of manuscript preparation.

*Western blot analysis.* Bradford assays were performed to determine total protein concentrations, which were normalized to 1 μg/μL for all samples. Samples were then prepared in sample buffer and heated to 95°C for 5 minutes. Samples were run on 10% to 12% polyacrylamide gels with the exception of 16% for cleaved caspases 3. Fifteen μL of protein lysates in sample buffer from each tissue were loaded within each well. Positive controls were loaded on each gel and represented lysates from U87 cells for phospho- and total PI3K, Akt, and p70S6K. Lysates from normal astrocytes were used as positive controls for cleaved caspase 3 expression. Gels were run at constant current (30mA) for 2 hours for maximum separation. Semi-dry transfer was then performed. The membrane was then blocked for 1 hour in 5% milk in 0.2% Tris buffer solution with TWEEN 20. The membranes were then washed in 0.2% TBST x 3 for 15 minutes each. The membranes were then incubated overnight with primary antibody directed against Total PI3K (Santa Cruz Biotechnology, Santa Cruz, CA), Total Akt (Cell Signaling Technology, Cambridge, MA), Total p70S6K (Santa Cruz Biotechnology), Phospho-PI3K (Cell Signaling Technology), Phospho-Akt (Cell Signaling Technology), Phospho-p70S6K (Cell Signaling Technology), and cleaved caspase 3 (Cell Signaling Technology). The residue targets for each phospho-specific antibody were: pAkt(Thr308), pp70S6K(Thr389), and pPI3K(Tyr508 of the p85 subunit), respectively. Subsequently, the membranes were washed in 0.2% TBST x 3 for 15 minutes each. Membranes were then incubated with secondary antibody for 45 minutes and subsequently washed. Chemiluminescent (Bio-Rad, Hercules, CA) detection was then used to detect expression levels of both total (combined phosphorylated and unphosphorylated) and

![Fig 1. Western blots measuring both phospho- and total levels of phosphatidylinositol 3-kinase (PI3K) pathway members.](image-url)

*Fig 1.* Western blots measuring both phospho- and total levels of phosphatidylinositol 3-kinase (PI3K) pathway members. There is a close association among activation patterns of PI3K, Akt, and p70S6K as measured by phosphorylated versions of these proteins. Glioblastomas (GBMs) have greater activation of PI3K pathway members than low-grade astrocytomas (LGAs). There are no detectable differences in total levels of these proteins based on histology.
phosphorylated versions of these proteins, the levels of which were quantitated using densitometry. To normalize among samples run on different gels, the expression levels were expressed as a ratio relative to that of the positive controls run on each gel. Representative Western blots are shown in Figure 1.

Statistical methods. The Fisher’s exact test was used to determine association between expression (as a binary variable) of phospho-PI3K, phospho-Akt and phospho-p70s6k with histopathologic glioma subtype (GBM v non-GBM). Correlation analysis of phospho-PI3K, phospho-Akt, and phospho-p70s6k among each other and with other covariates, was performed using Spearman correlation coefficient. Expression was analyzed both as a dichotomous covariate indicating whether expression was detectable (>0) and as the continuous value of the expression level. The results are reported as statistically significant if a two-sided P value was less than .05. Survival curves were calculated using the Kaplan-Meier method (Fig 2). To further graphically display the relationship between phospho-PI3K, phospho-Akt, and phospho-p70s6k levels with survival, both censored and uncensored cases were selected, and a scatterplot was presented of the survival times by phospho-PI3K, phospho-Akt, and phospho-p70s6k levels, respectively, for each subject (Fig 3).

**RESULTS**

Expression Patterns of Total PI3K, Akt, and p70s6k in GBM Versus Non-GBM Tumors

The proportion of patients with detectable expression of each respective phospho-protein were: phospho-PI3K (52 of 92 patients, overall; 10 of 36 non-GBM patients; 42 of 56 GBM patients); phospho-Akt (50 of 92 patients, overall; 13 of 36 non-GBM patients; 37 of 56 GBM patients); phospho-p70s6k (36 of 92 patients, overall; five of 36 non-GBM patients; 31 of 56 GBM patients). The median expression levels are: phospho-PI3K = 0.2375 (range, 0 to 2.85); phospho-Akt = 0.1095 (range, 0 to 2.04); phospho-p70s6k = 0 (range, 0 to 2.03). There were no statistically significant differences in expression of total levels of either PI3K, Akt, or p70s6k in GBM versus non-GBM tumors (illustrated in Fig 1). This suggests that any differences in phosphorylated versions of these proteins, indicative of increased signaling activity through these pathways is unlikely to be secondary.
to expression levels of these proteins or genomic amplification. Rather, any differences in activation status of these proteins would be more likely secondary to events at the signal transduction level.

**Expression Patterns of Phospho-PI3K, Phospho-Akt, and Phospho-p70\textsuperscript{66k} in GBM Versus Non-GBM Tumors**

Activation states of these proteins can be measured and quantitated by Western analysis using antibodies that detect only the phosphorylated versions of these respective proteins. Figure 1 demonstrates representative Western blots illustrating activation patterns of these PI3K pathway members and their relationship to one another, as well as to histopathologic classification. It was determined that the phosphorylated versions of all three proteins were more commonly expressed in GBM versus non-GBM tumors (Table 2). On further analysis of the non-GBM cases that expressed phospho-PI3K, there was a significantly greater representation of anaplastic cases (e.g., anaplastic astrocytoma, anaplastic oligodendroglioma, or anaplastic mixed tumors) compared to low-grade tumors taken in aggregate ($P = .026$). This indicates that activation of PI3K pathway may be a later rather than an earlier event in gliomagenesis. Further, it was determined that the quantitatively-determined expression levels (as measured as continuous variables) of the activated version of PI3K, p70\textsuperscript{66k}, and Akt were significantly higher in GBM versus non-GBM tumors ($P < .0001$, $P < .0001$, and $P = .004$, respectively).

As Akt and p70\textsuperscript{66k} are known to be important downstream mediators of PI3K, it would be expected that there would be a significant correlation among the activation states of all three proteins, as predicted by preclinical models. Indeed, this is what was observed between levels of phospho-PI3K with phospho-Akt ($\rho = 0.612; P < .0001$) and with phospho-p70\textsuperscript{66k} ($\rho = 0.626; P < .0001$), respectively, and between levels of phospho-Akt and phospho-p70\textsuperscript{66k} ($\rho = 0.460; P < .0001$).
Association of Phospho-PI3K, Phospho-Akt, and Phospho-p70S6K Expression With Outcome

The degree of cellular apoptosis of frozen glioma specimens was assessed by measuring levels of cleaved caspase 3, which is a known effector caspase whose activation is required for apoptosis. Activation of caspase 3 can be reliably measured by assessing levels of its cleaved form, which is a reliable biomarker for apoptosis. Available preclinical data suggests that PI3K family members are involved in suppression of apoptosis. This hypothesis was tested in the 92 frozen glioma specimens. Indeed, there was a significant inverse relationship found between levels of cleaved caspase 3 and levels of phospho-PI3K (P = 0.267; 95% CI, 0.076 to 0.940), and histology (P = 0.807; 95% CI, 0.651 to 1.000) remained significantly associated with adverse outcome (Table 3). Next, we examined the prognostic significance of expression of these phospho-proteins restricted only to the subset of GBM patients. As all of these 56 patients were treated by a combination of surgery with postoperative radiation (to 60 Gy in 2 Gy fractions) as the only adjuvant therapy, any association of phospho-PI3K family members with adverse survival would be highly suggestive that this pathway may play an important role in radiation resistance. Indeed, it was found that expression of all three phospho-proteins was associated with adverse outcome in the subset of GBM patients (phospho-PI3K, P = .0021; phospho-Akt, P = .0099). Figure 4 demonstrates corresponding Kaplan-Meier survival curves for GBM patients only stratified by expression of these respective phospho-proteins.

DISCUSSION

Given the aggressive clinical behavior of human gliomas, much effort has been dedicated into investigating the molecular features that contribute to the aggressive phenotypes of these tumors. Gene expression analysis on the transcriptional level appears to be a promising approach and has been found to have greater prognostic value than histopathology alone. Chromosomal alterations have also been

Table 2. Frequency of Activation of PI3K Pathway Members in GBM Versus Non-GBM Tumors

<table>
<thead>
<tr>
<th>Marker</th>
<th>GBM Tumors (n = 56)</th>
<th>Non-GBM Tumors (n = 36)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No. of Patients %</td>
<td>No. of Patients %</td>
<td></td>
</tr>
<tr>
<td>Phospho-PI3K</td>
<td>42 75</td>
<td>10 28</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Phospho-p70S6K</td>
<td>31 56</td>
<td>5 14</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Phospho-Akt</td>
<td>37 66</td>
<td>13 36</td>
<td>.0049</td>
</tr>
</tbody>
</table>

Abbreviations: PI3K, phosphatidylinositol 3-kinase; GBM, glioblastoma multiforme.

Table 3. Multivariate Analysis of Overall Survival Stratified by Expression Levels of Phospho-PI3K Members and Cleaved Caspase 3

<table>
<thead>
<tr>
<th>Variable</th>
<th>Hazard Ratio</th>
<th>95% CI for Hazard Ratio</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Histology</td>
<td>0.807</td>
<td>0.651 to 1.000</td>
<td>.039</td>
</tr>
<tr>
<td>Phospho-PI3K level</td>
<td>4.703</td>
<td>1.758 to 12.587</td>
<td>.0021</td>
</tr>
<tr>
<td>Phospho-Akt level</td>
<td>1.033</td>
<td>0.562 to 1.896</td>
<td>.917</td>
</tr>
<tr>
<td>Phospho-p70S6K</td>
<td>11.543</td>
<td>3.818 to 34.896</td>
<td>&lt;.0001</td>
</tr>
<tr>
<td>Cleaved caspase</td>
<td>0.267</td>
<td>0.076 to 0.940</td>
<td>.0397</td>
</tr>
</tbody>
</table>

Abbreviation: PI3K, phosphatidylinositol 3-kinase.
found to be of prognostic significance in anaplastic oligodendrogliomas. However, gene expression/genomic alterations may not always reflect events at the signaling transduction level. As signal transduction mediators directly contribute to important cellular behavior such as proliferation, motility, invasion, angiogenesis, and survival, identifying deregulated pathways and mediators at this level may prove to be of important therapeutic value.

In this study, we have examined the prognostic value of activation states of a key signaling pathway in gliomas: the PI3K/Akt/p70s6k cascade. The PI3K pathway can be upregulated in gliomas through several mechanisms. Perhaps the most common known mechanisms are through mutations or loss of heterozygosity of phosphatase and tensin homolog (PTEN) or through amplification/overexpression of critical growth factors such as EGFR. It has been recently reported by Mischel et al through immunohistochemical analysis of 45 GBM cases on tissue microarrays that loss of PTEN is closely associated with activation of Akt. Further, it was demonstrated that expression of the EGFRvIII mutant is an alternate mechanism of Akt activation in the presence of wild-type PTEN. There remains, however, a relative paucity of correlative data of PI3K activation patterns in the various stages of gliomagenesis and association with clinical outcome, which were the primary objectives of this study.

Since it has been well-documented that higher-grade gliomas have a more aggressive clinical course than their lower-grade counterparts, our first objective was to determine whether patterns of activation differed significantly between the highest grade tumors (eg, GBMs), compared with lower-grade tumors (eg, non-GBMs). Our finding that the frequency of expression of activated versions of these proteins is significantly higher in GBM versus non-GBM tumors suggests that the PI3K pathway is activated at a relatively late stage of gliomagenesis. Further, of the non-GBMs that demonstrated activation of PI3K, anaplastic tumors had a significantly higher percentage of expressing phospho-PI3K than their nonanaplastic counterparts, further supporting a progressive activation pattern with increasing grade.

Fig 4. Expression (bottom dashed line in each graph) of (A) phosphatidylinositol 3-kinase ($P < .0002$), (B) phospho-p70s6k ($P < .0001$), and (C) phospho-Akt ($P < .0099$) is associated with significantly reduced overall survival times when only the subset of glioblastoma patients are considered. Expression is defined here as a binary variable (positive vs negative).
When considering only the subset of tumors expressing activated, or phosphorylated, variants of PI3K family members, it was found that GBMs had significantly higher levels of phospho-PI3K and phospho-p70s6k, but not of phospho-Akt, compared to their non-GBM counterparts. This observation implies that GBMs may have greater reliance on these pathways for critical functions (survival, proliferation, invasion, angiogenesis, and so on) than lower grade tumors. Our preclinical data suggests that the degree of PI3K pathway activation is significantly associated with radiation resistance. The finding here that phospho-PI3K, phospho-Akt, and phospho-p70s6k levels are inversely associated with cleaved caspase 3 levels, which is a marker of apoptosis, suggests that PI3K members may act, at least in part, through suppression of apoptosis, thereby providing a possible mechanism by which the PI3K pathway may promote the resistance to radiation therapy in GBMs, as reported in this series. As our preclinical data and that of others suggest that survival in normal brain cells is closely dependent on PI3K activation, identifying tumor-specific upstream and downstream mediators of PI3K may prove to be of greater therapeutic gain than inhibiting PI3K itself. Further, pathways downstream of PI3K such as p70s6k can be likewise activated by PI3K- and Akt-independent mechanisms. Therefore, to attain significant therapeutic gain in the treatment of malignant gliomas may ultimately require simultaneous inhibition of multiple signaling pathways. Presently, cooperative group studies are investigating the safety and efficacy of using small molecule inhibitors that downregulate PI3K activity either upstream (e.g., gefitinib) or downstream (e.g., Rapamycin, CCI-779) either alone or in combination with radiation. Initial results suggest that there may indeed be some clinical activity of anti-EGFR agents in gliomas. The results of these studies will ultimately reveal whether PI3K pathway members can be useful therapeutic targets, as well as of prognostic value, in human gliomas.

**Authors' Disclosures of Potential Conflicts of Interest**

The authors indicated no potential conflicts of interest.

---

**REFERENCES**

29. Harada H, Andersen J, Mann M, et al: P70S6 kinase signals cell survival as well as cell growth, inactivating the pro-apoptotic molecule...
BAD. Proc Natl Acad Sci U S A 98:9666-9670, 2001