Abstract  
Purpose: Glioblastoma is the most common primary malignant tumor in the brain. It aggressively invades the surrounding parenchyma, often allowing the tumor to progress after surgery. Accumulating evidence has shown that phosphorylated p21-activated kinase 1 (Pak1), a mediator of small guanosine triphosphatases, plays a role in the proliferation, survival, and invasiveness of cancer cells. Thus, we examined patterns of Pak1 expression in glioblastoma and sought to determine whether the level of phosphorylated Pak1 in glioblastoma cells is associated with patient survival time.

Experimental Design: We carried out immunohistochemical staining for phosphorylated Pak1 in tumor specimens from 136 patients with glioblastoma; the tumors were classified according to Pak1 protein levels in the cytoplasm and nucleus. We compared the patients’ overall survival times using Kaplan-Meier analysis and estimated the effects of levels of cytoplasmic or nuclear phosphorylated Pak1. We then down-regulated Pak1 by using small interfering RNA to knock down Pak1 in two glioblastoma cell lines to determine whether Pak1 contributed to cell viability and invasion.

Results: Median overall survival was significantly shorter in patients with tumors showing a moderate or high level of cytoplasmic phosphorylated Pak1 than in patients with tumors showing no cytoplasmic phosphorylated Pak1. The level of nuclear phosphorylated Pak1 was not related to survival time. Knockdown of Pak1 suppressed the invasion, but not the viability, of U87-MG and U373-MG cells.

Conclusions: The presence of phosphorylated Pak1 in the cytoplasm of glioblastoma cells is associated with shorter survival, and Pak1 plays a role in the invasiveness of glioblastoma. These data suggest that Pak1 might be a potential target for the management of glioblastoma.

Glioblastoma is typically a very aggressive tumor and the deadliest of brain cancers. It is also the most common primary malignant tumor in the brain. Even with the current frontline combination therapy consisting of surgery, radiation, and temozolomide, the median survival time is only 15 months (1). Although glioblastoma rarely metastasizes to distant organs, it typically aggressively invades the surrounding parenchyma; after surgery, most recurrent tumors arise within 3 cm of the resection cavity and eventually become fatal (2, 3). Histologic examinations have revealed that glioma cell invasion takes place along myelinated fiber tracts and along the basement membrane of blood vessels. Tumor cell invasion is a dynamic and orchestrated process that involves the translocation of neoplastic cells through host cellular and extracellular matrix barriers. A three-step theory of invasion is generally acknowledged (2, 4): first, tumor cells attach to extracellular matrix–related proteins through receptor-mediated adhesion; second, tumor-secreted proteases degrade the extracellular matrix; and third, the cytoskeleton is rearranged and the tumor cell moves. Recent studies have shown that members of the Rho family of small guanosine triphosphatases (GTPases) are key regulators of glioma invasion in vitro and that they regulate invasion by rearranging actin filaments in the cytoskeleton through signal transduction pathways that are activated by membrane receptors (5–8). However, details of the mechanisms by which Rho GTPases facilitate invasion of malignant glioma cells are not fully understood, nor has the association between the status of Rho GTPases and the survival of patients with glioblastoma been clearly documented.

p21-activated kinase 1 (Pak1) is a serine/threonine protein kinase that regulates cytoskeletal remodeling and cell motility.
through actin and microtubules (9, 10). Pak1 was initially determined to be activated by Rho GTPases Rac and Cdc42 and to be an important mediator of these enzymes in the brain (11). Pak1 has various phosphorylation substrates and interacting proteins; thus, it plays multiple roles in both the nucleus and cytoplasm. In the nucleus, Pak1 activates transcription factors such as activator protein 1 and nuclear factor κB (12, 13). In the cytoplasm, Pak1 interacts with or phosphorylates several proteins, including merlin, filamin, and vimentin, resulting in modulation of the cytoskeleton.

Recently, accumulating evidence has shown that Pak1 plays a role in proliferation, survival, and invasiveness of cancer cells (10). For example, Pak1, together with endogenous and hyperactive Rac3, controls proliferation of breast cancer cells (14). Pak1 plays an important role in cell survival pathway by inhibiting the proapoptotic effect of Bad (15) or the activation of caspases (16). In addition, overexpression of dominant-negative Pak1 suppressed the invasive phenotype in breast cancer cells, whereas overexpression of constitutively active Pak1 increased their anchorage-independent growth and invasiveness (12, 17). Transgenic mice that had constitutively active Pak1 in mammary gland specimens exhibited hyperplasia in the mammary epithelium and eventually developed mammary gland tumors (18, 19). Pak1 overexpression has also been detected in breast and colon cancer samples and Pak1 gene amplification has been found in ovarian and bladder cancers. Furthermore, Pak1 has been associated with tamoxifen resistance in breast cancer tissues (20). However, whether Pak1 plays a role in glioblastoma has yet to be determined.

In the study reported here, we examined the possibility that Pak1 activation is associated with shorter patient survival in case of glioblastoma. We did this by first determining whether phosphorylated Pak1 was expressed in glioblastoma tissues by immunohistochemical staining with an antibody specific to Pak1 phosphorylated on Thr212: this site was chosen because phosphorylation at Thr212 has been reported to be associated with neurite growth and the motility of developing neurons. The tissues were then classified according to the intensity and localization of immunoreactivity to the antibody and analyzed for statistical associations between staining patterns and patient survival time. Our results constitute the first reported evidence for statistical associations between staining patterns and patient survival. Our results were filled with 2 mL of 10% fetal bovine serum–containing DMEM. After 72 h after the cells were transfected with or without control or Pak1 siRNA, they were exposed to 10 μL of WST-1 reagent for 1 h at 37°C. The absorbance at 450 nm was measured in a microplate reader. The viability of cells treated with vehicle alone was considered to be 100% and was used as the reference value.

Cell viability assay. The cytotoxic effect of Pak1 siRNA on cultured glioma cells was determined by using cell proliferation reagent WST-1 (Roche Applied Science) as described previously (24). Cells were seeded at 3 × 10^4 per well in a 96-well plate and incubated at 37°C for 24 h. At 72 h after the cells were transfected with or without control or Pak1 siRNA, they were exposed to 10 μL of WST-1 reagent for 1 h at 37°C. The absorbance at 450 nm was measured in a microplate reader. The viability of cells treated with vehicle alone was considered to be 100% and was used as the reference value.

Matrigel invasion assay. The invasion assay was done using a six-well plate transwell insert with 8-μm pore size filters (Fisher Scientific) coated with Matrigel (BD Biosciences). U87-MG and U373-MG cells were transfected with siRNA as described above. Twenty-four hours after transfection, 5 × 10^5 cells were placed into each transwell insert, and then 1 mL of serum-free medium was added. The lower chambers were filled with 2 mL of 10% fetal bovine serum—containing DMEM. After 72 h of incubation at 37°C, noninvasive cells on the top surface of the filter were wiped off using cotton swabs. Invasive cells on the lower surface of the filter were fixed and stained with the Hema-3 staining system (Fisher) according to the manufacturer’s instructions. Cells were counted in five fields for each filter under a microscope.

Statistical analysis. The number of replicate experiments per condition was determined by the between-replicate variation and the size of difference to be considered statistically significant. We believed that for the in vitro assays, a clear-cut difference would be at least 3 SD.

Materials and Methods

Study population. We retrospectively reviewed tissue specimens and patient records for 136 patients who had been newly diagnosed with glioblastoma (WHO glioma grade 4) at The University of Texas M. D. Anderson Cancer Center (Houston, TX) from 1993 to 2004. Our inclusion criteria for the samples required that (a) the patients had not received any prior therapy for the tumor; (b) the archival paraffin-embedded tissues were available for immunohistochemical staining; and (c) the cases were reviewed by a neuropathologist to ensure that they fulfilled histologic criteria for glioblastoma using current WHO guidelines, which include a high-grade astrocytic tumor with microvascular proliferation and/or necrosis. The research protocol was approved by the M. D. Anderson Cancer Center Institutional Review Board. The median age and survival time for patients in our study population were 56 years and 54 weeks, respectively (by Kaplan-Meier estimates). Survival is reported as the number of weeks after the initial surgery date.

Immunohistochemical analysis. A monochonal antibody to Pak1 phosphorylated on Thr212 was obtained from Sigma Chemical. Immunohistochemical analysis was done as previously described (21) and the slides were incubated with primary antibody at a dilution of 1:1,000 in PBS at 4°C overnight. Cytoplasmic and nuclear staining was scored using a three-tiered system as previously described (22), with scoring done for the most highly stained area in the tumor section. Briefly, a score of 2 meant strong positive staining in the majority of tumor cells in at least one medium-power (100×) microscopic field; a score of 1 meant weak/patchy staining in the tumor cells; and a score of 0 meant no staining. The investigator was blinded to clinical data when the staining was scored. As a quality assurance process, 10% of all the slides were randomly selected and reexamined independently by two other observers.

Cell culture. Human glioblastoma cell lines U87-MG and U373-MG were purchased from the American Type Culture Collection. Cells were cultured in DMEM supplemented with 10% fetal bovine serum (Invitrogen), 100 units/mL penicillin (Invitrogen), and 2.5 μg/mL fungizone (Invitrogen) at 37°C in 5% CO2.

siRNA transfections. Pak1 siRNA was purchased from Cell Signaling Technology (18). SiCONTROL non-targeting siRNA (Dharmacon, Inc.) was used as the control siRNA. Cells were transfected with siRNA using Oligofectamine Transfection Reagent (Invitrogen) for 72 h according to the manufacturer’s instructions. The final concentration of siRNA was 100 nmol/L.

Western blotting. For Western blotting, soluble proteins were isolated from U87-MG and U373-MG cells transfected with or without control or Pak1 siRNA for Western blotting, as described previously (23). Equal amounts of protein were separated by 15% SDS-PAGE (Bio-Rad) and transferred onto a Hybond-P membrane (Amersham). The membranes were treated with antibodies to total Pak1 (Cell Signaling) and β-actin (Sigma) overnight at 4°C and incubated for 1 h with a horseradish peroxidase–conjugated antiserum or antirabbit secondary antibody (1:3,000 dilution; Amersham) at room temperature for 1 h. Bound antibody complexes were detected using an enhanced chemiluminescence reagent (Amersham) according to the manufacturer’s instructions.

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Statistical analysis. The number of replicate experiments per condition was determined by the between-replicate variation and the size of difference to be considered statistically significant. We believed that for the in vitro assays, a clear-cut difference would be at least 3 SD.
in magnitude. For example, if the SD in percentage apoptosis is ~2%, a 3-SD difference between conditions would equate to a 6% difference. Four replicates would be required to detect a 3-SD difference between conditions, assuming the use of a two-sided, independent-group, equal-variance Student's t test with \( x = 0.5\% \) and \( \beta = 10\% \). The data were expressed as mean \( \pm \) SD. The significance of the experiments was determined by using the Student t test (two tailed). \( P < 0.05 \) was considered statistically significant. Kaplan-Meier analysis was used to estimate overall survival. Cox proportional hazards regression analysis was used for estimating hazard ratios (HR) and associated 95% confidence intervals (95% CI). SPLUS-2000 (MathSoft, Inc.) was used for these analyses. Multivariate models were fit by use of a Cox proportional hazards regression model (22). Patients who were still alive at our last contact were considered a censored event in analysis.

Results

Immunohistochemical staining for phosphorylated Pak1 and survival analysis. The patient characteristics and clinical features of their glioblastomas are summarized in Table 1. The cytoplasmic and nuclear staining of the glioblastomas was scored according to the intensity of the immunohistochemical staining with an antibody to Pak1 phosphorylated on Thr\(^{12}\). Among 136 glioblastoma tissues, 41 had a score of 0 for cytoplasmic staining (designated as C0), 49 a score of 1 (C1), and 46 a score of 2 (C2); 3 had a score of 0 for nuclear staining (N0), 23 a score of 1 (N1), and 110 a score of 2 (N2). Representative samples of different combinations of cytoplasmic and nuclear staining intensity are shown in Fig. 1. Additionally, 10 samples of normal brain tissues were stained and scored: no cytoplasmic staining and no or only weak nuclear staining for phosphorylated Pak1 were detected in any of the normal tissues (C0N0; Fig. 1).

To determine whether cytoplasmic or nuclear phosphorylated Pak1 was associated with patient survival time, we carried out Kaplan-Meier survival analysis. The Kaplan-Meier survival curves for patients subdivided by cytoplasmic phosphorylated Pak1 score are shown in Fig. 2A. For the 41 patients with C0 staining, the median overall survival time was 69 weeks; for the 49 patients with C1 staining, the median overall survival time was 46 weeks; and for the 46 patients with C2 staining, the median overall survival time was 56 weeks [HR for C1 versus C0, 1.7 (95% CI, 1.1-2.7), \( P = 0.023 \); HR for C2 versus C0, 1.7 (95% CI, 1.1-2.7), \( P = 0.028 \)]. Thus, the median survival time of patients with C1 and C2 staining was significantly shorter than that of patients with C0 staining.

In contrast, the association between survival time and amount of nuclear phosphorylated Pak1 was unclear (Fig. 2B). Because very few patients \( (n = 3) \) had N0 staining, this group was excluded from the statistical analysis. For the 23 patients with N1 staining, the median overall survival time was 41 weeks; for the 110 patients with N2 staining, the median overall survival time was 55 weeks (HR, 1.3; 95% CI, 0.8-2.1; \( P = 0.37 \)).

As this was not a prospective study, we did not compute power and sample size ahead of time (because the prevalence of the markers was unknown). However, the power of the comparisons can be inferred via CI. For the cytoplasmic values, CI excluded 1.0 with a relatively modest point estimate of 1.7. Thus, power was adequate for these comparisons (also there were more than 40 patients in each group). However, for nuclear values, CI included both 1.0 and 2.0, indicating that the data are consistent with both no difference and a 2-fold difference, which suggests that the data are not sufficiently powerful to detect a 2-fold difference as significant. In addition, there are only 23 patients in one of the groups.

Then, we conducted the multivariate analyses. In cytoplasmic staining, after adjustment for age, Karnofsky performance scale, and extent of resection, HR for C1 versus C0 was 1.5 (95% CI, 0.9-2.4; \( P = 0.12 \)) and HR for C2 versus C0 was 1.5 (95% CI, 0.9-2.5; \( P = 0.085 \)). In nuclear staining, after adjustment for age, Karnofsky performance scale, and extent of resection, HR was 1.3 (95% CI, 0.8-2.3; \( P = 0.28 \)).

These results indicate that a high level of phosphorylated Pak1 in the cytoplasm, but not in the nucleus, is significantly associated with shorter overall survival in patients with glioblastoma.

Effect of Pak1 knockdown with siRNA. To determine the role of Pak1 in glioblastoma cells, we used siRNA technology to

Table 1. Patient characteristics by cytoplasmic staining score for phosphorylated Pak1

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Total, N (%)</th>
<th>p-Pak1 C0, n (%)</th>
<th>p-Pak1 C1, n (%)</th>
<th>p-Pak1 C2, n (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total</td>
<td>136 (100)</td>
<td>41 (30)</td>
<td>49 (36)</td>
<td>46 (34)</td>
</tr>
<tr>
<td>Age (y)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>56; mean, 56</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;60</td>
<td>83 (61)</td>
<td>28 (68)</td>
<td>28 (57)</td>
<td>27 (59)</td>
</tr>
<tr>
<td>≥60</td>
<td>53 (39)</td>
<td>13 (32)</td>
<td>21 (43)</td>
<td>19 (41)</td>
</tr>
<tr>
<td>KPS score</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>&lt;90</td>
<td>65 (48)</td>
<td>19 (46)</td>
<td>26 (53)</td>
<td>20 (43)</td>
</tr>
<tr>
<td>≥90</td>
<td>71 (52)</td>
<td>22 (54)</td>
<td>23 (47)</td>
<td>26 (57)</td>
</tr>
<tr>
<td>Surgery</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>GTR</td>
<td>72 (53)</td>
<td>27 (66)</td>
<td>21 (43)</td>
<td>24 (52)</td>
</tr>
<tr>
<td>STR</td>
<td>61 (45)</td>
<td>12 (29)</td>
<td>27 (55)</td>
<td>22 (48)</td>
</tr>
<tr>
<td>Biopsy</td>
<td>3 (2)</td>
<td>2 (5)</td>
<td>1 (2)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Survival (wk)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Median</td>
<td>54</td>
<td>69</td>
<td>46</td>
<td>56</td>
</tr>
</tbody>
</table>

Abbreviations: CX, score of cytoplasmic staining for Pak1; KPS, Karnofsky performance scale.
knock down Pak1. Transfection of U87-MG and U373-MG cells with Pak1 siRNA reduced the expression of Pak1 protein by >50%, whereas control siRNA did not show much effect (Fig. 3A). Because Pak1 siRNA has been reported to inhibit tumor growth and induce apoptosis in breast cancer cells (18), we examined the effect of Pak1 siRNA transfection on the growth of cells in the two malignant glioma lines. Unexpectedly, treatment with Pak1 siRNA for 72 h did not reduce the viability of either cell type (Fig. 3B).

Because several studies reported that Pak1 plays an important role in tumor invasion by modulating the cytoskeleton (12, 25, 26), we next examined the effect of Pak1 siRNA on tumor cell invasion in U87-MG and U373-MG glioma cells. Treatment with Pak1 siRNA for 72 h significantly inhibited tumor cell invasion by 69 ± 10% in U87-MG cells and by 40 ± 23% in U373-MG cells (Fig. 3C and D; P < 0.05 for both cell types). These results collectively suggest that Pak1 mediates invasion, but not tumor cell growth, in both these glioblastoma cell lines.

**Discussion**

The present study yielded what we believe is the first evidence that phosphorylated Pak1 in the cytoplasm, but not in the nucleus, is significantly associated with a reduction in the median overall survival time of patients with glioblastoma. Furthermore, partial knockdown of Pak1 decreased the invasion, but not the proliferation, of glioblastoma cells. These results indicate the association between phosphorylated Pak1 and decreased survival time of patients with glioblastoma for the first time.
For immunohistochemical staining analysis, we used an antibody to Pak1 phosphorylated on Thr^{212}, which is located in the protein regulatory domain. Thr^{212} of Pak1 is phosphorylated by cyclin-dependent kinase 5 and its neuron-specific regulator, p35; these Rac effectors were originally reported to inhibit Pak1 activity (27). However, phosphorylation on Thr^{212} was later proved not to directly affect Pak1 activity but to modulate the interaction of Pak1 with other proteins, resulting in the promotion of cell spreading after mitosis (28–30). Pak1 phosphorylated on Thr^{212} is also known to play an important role in neuronal development: it modifies the cytoskeleton and enhances neurite outgrowth and neuron migration (27, 28). In line with these findings, our results using Pak1 siRNA showed that knockdown of Pak1 inhibited the invasiveness of glioblastoma cells (Fig. 3C and D). Although glioblastoma is generally considered to be derived from glia, not from neurons, recent studies have shown that glioma stem cells have characteristics similar to those of neural stem cells, such as a capability for differentiating into multiple cell types (including neurons) and the ability to migrate into the brain (31–33). Thus, we speculate that Pak1 may regulate the motility and invasiveness of glioblastoma stem cell, an idea that warrants further investigation.

Although the phosphorylation of Pak1 at a different residue, Thr^{423}, is directly associated with Pak1 activation, there is no suitable antibody available for immunohistochemical analysis of Pak1 phosphorylation at this site. Thus, previous investigators have had to use antibodies to total Pak1 or Pak1 phosphorylated at Thr^{212} for immunohistochemical staining of cancer tissues. Tests with anti-total Pak1 antibody have shown overexpression of Pak1 in several cancer tissues including breast, colon, and ovarian cancers (17, 34–36). One study showed that Pak1 overexpression was often associated with Pak1 activity and invasiveness in breast cancer cells and tissues (17). In a preliminary study, we carried out immunohistochemical staining of glioblastoma tumor tissues for total Pak1 but did not find any significant association between the Pak1 expression level and patient survival time (data not shown).

A recent study revealed that the level of nuclear Pak1 phosphorylated on Thr^{212} increased with the malignancy of mammary tumors in transgenic mice that had constitutively active Pak1 in their mammary glands (19). Another in vitro study of breast cancer tissues showed that Pak1 up-regulated cyclin D1 through nuclear factor-κB activation and thereby enhanced the proliferation of breast cancer cells (35). These studies emphasize the importance of Pak1 in the nucleus, not just in the cytoplasm, in breast cancer. Similarly, in the present study, we showed that glioblastoma cells and tissues had higher levels of phosphorylated Pak1 than cultured astrocytes (data not shown) and normal brain specimens, respectively. However, we found that only phosphorylated Pak1 in the cytoplasm, not in the nucleus, was associated with shorter patient survival time. Nevertheless, our findings do not exclude the possibility that Pak1 has a role in the nucleus in glioblastoma; almost all the glioblastoma tissues we studied (133 of 136) were positive for phosphorylated Pak1 in the nucleus, whereas normal brain showed no or only moderate staining. It is possible that nuclear Pak1 plays a role in some process common to most glioblastoma (such as oncogenesis) but absent from less aggressive tumors (e.g., low-grade gliomas) and normal tissues.

Although few published studies have directly associated Pak1 with glioblastoma cells or tissues, several studies have shown that the migration or invasion of glioblastoma cells is regulated by the Rho family of GTPases; this finding suggests the involvement of Pak1, which mediates these GTPases (5–8). siRNA-induced inhibition of Rac1, a member of the Rho family of GTPases, inhibited the migration and invasion of glioblastoma cells but did not inhibit the proliferation and survival of these cells (7); these findings support ours. Another line of study suggests a link between Pak1 and a key signaling pathway in glioblastoma: the epidermal growth factor receptor/phosphatidylinositol 3-kinase/Akt pathway. Up-regulation of epidermal growth factor receptor and deficiency of phosphatase and tensin homologue, both of which activate the epidermal growth factor receptor/phosphatidylinositol 3-kinase/Akt pathway, are the molecular signatures of glioblastoma (37, 38).
It has also been shown that epidermal growth factor receptor, phosphatidylinositol 3-kinase, and Akt phosphorylate or associate with Pak1, transducing upstream signals to effector molecules (39–41). Thus, some effects of this pathway, especially cell migration and invasion, may be mediated by Pak1.

In summary, we showed that phosphorylated Pak1 in the cytoplasm of glioblastoma cells is significantly associated with shorter median patient survival time. Knockdown of Pak1 using siRNA inhibited the cell invasion, but not the cell viability, of glioblastoma cells. These findings represent an opportunity for therapeutic targeting. Because Pak1 plays a putative role in apoptosis (15, 16), we would not expect an effect of Pak1 inhibition on survival under normal growth conditions but rather a synergistic effect when combined with radiation and/or chemotherapy such as temozolomide, which causes nonapoptotic autophagic cell death (42). Further studies must be done to verify our findings and to determine the details of the molecular pathways in which Pak1 plays a part, which potentially could then be exploited for therapeutic benefit.

Acknowledgments

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References


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Phosphorylated Pak1 Level in the Cytoplasm Correlates with Shorter Survival Time in Patients with Glioblastoma

Hiroshi Aoki, Tomohisa Yokoyama, Keishi Fujiwara, et al.

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