The signaling pathways that underlie the pathogenesis of pediatric gliomas are poorly understood. We characterized the PI3K/Akt/mTOR pathway in pediatric gliomas of all grades. Using immunohistochemistry, we assessed activation of the PI3K/Akt/mTOR pathway by evaluating the downstream signaling molecules phospho(p)-S6, phospho(p)-4EBP1, and phospho(p)-PRAS40; PTEN; and PTEN promoter methylation, as well as the MIB labeling index. We correlated these findings with the clinical outcomes of 48 children with gliomas. Eighty percent of high-grade gliomas (12/15) showed activation of the PI3K/Akt/mTOR pathway based on p-S6 and p-4EBP1 expression. The majority of high-grade gliomas were negative for PTEN expression (10/15), and 50% had PTEN promoter methylation (grade III: 2/4; grade IV: 3/6). Low-grade gliomas demonstrated PI3K/Akt/mTOR pathway activation in 14/32 (43.8%) by p-S6 and 16/32 (50%) by p-4EBP1. Over 50% of grade I (6/11) and almost all grade II tumors (6/7) showed PTEN promoter methylation. Tumor grade correlated negatively with PTEN expression and positively with expression of p-S6 and p-4EBP1 (PTEN: \(P = .0025\); pS6: \(P = .0075\); p-4EBP1: \(P = .0066\)). There was a trend toward inverse correlation of methylation of the PTEN promoter with expression of PTEN protein (\(P = .0990\)) and direct correlation of expression of p-S6 and p-4EBP1 with poorer clinical outcome, as measured by progression-free survival (p-S6: \(P = .0874\); p-4EBP1: \(P = .0475\)). Tumors with no PTEN expression had a higher MIB labeling index (\(P = .007\)). The majority of pediatric gliomas show activation of the PI3K/Akt/mTOR pathway, with methylation of the PTEN promoter occurring commonly in these tumors.

Keywords: pediatric gliomas, PI3K/Akt/mTOR, PTEN promoter methylation.
associated with clinical outcome in a large series of PAs but portended favorable outcome in a second cohort of incompletely resected PAs.4

In this study, we explored the role of the phosphatidylinositol 3-kinase (PI3K)/Akt/mammalian target of rapamycin (mTOR) signaling pathway in pediatric gliomagenesis. In adult gliomas, PI3K/Akt/mTOR is commonly activated through various mechanisms, including alterations in the tumor suppressor protein phosphatase and tensin homolog (PTEN). Whereas mutation of PTEN is common in de novo malignant adult gliomas,5,8,9 methylation of the PTEN promoter is an underlying mechanism of PTEN alteration found in adult LGGs and secondary malignant gliomas,8 likely leading to PI3K activation and tumor transformation and growth.8,9 In contrast to adult gliomas, PTEN mutations are rare in pediatric gliomas and other mechanisms of PI3K/Akt/mTOR activation remain largely unknown. The alternative mechanisms of PI3K/Akt/mTOR activation in pediatric gliomas may have important clinical implications. For example, the finding that PI3K/Akt/mTOR is activated due to PTEN promoter methylation in adult LGGs coupled with the unique sensitivity of tumors with activated PI3K/Akt-to-mTOR inhibitors has led to a promising clinical trial of everolimus (RAD001) for recurrent adult LGGs. Because of these data, we analyzed the prevalence of PTEN promoter methylation and activation of the PI3K/Akt/mTOR pathway in pediatric gliomas and their associations with clinical outcome.

Methods

Patients and Tissue Samples

Frozen tissue and formalin-fixed, paraffin-embedded sections from 32 newly diagnosed pediatric LGGs (25 WHO grade I and 7 WHO grade II) and 16 high-grade gliomas (HGGs) (7 WHO grade III and 9 WHO grade IV; no diffuse intrinsic pontine gliomas were included in this study) were obtained from the University of California, San Francisco (UCSF) Brain Tumor Research Center tissue bank, under appropriate institutional review board approval. The cohort included 17 girls and 31 boys. The age at diagnosis was from 1 month to 18 years of age. One patient was diagnosed with neurofibromatosis type 1 and none had tuberous sclerosis. Samples were collected from 1987 through 2007. Clinical information was obtained from medical record reviews. Survival data were collected through the UCSF Cancer Registry.

DNA Preparation and Bisulfite Treatment

DNA isolation and bisulfite treatment were carried out for samples for which frozen tissues were available, as previously described.3 Briefly, genomic DNA was isolated from approximately 25 mg wet weight of frozen tissue using the QIAamp DNA Mini Kit (Qiagen) according to the manufacturer’s instructions. One microgram of purified DNA was diluted in 36 μL H2O, 4 μL of 3.0 M NaOH was added, and the DNA was denatured at 37°C for 15 min. The samples were then treated with 416 μL of 3.6 M sodium bisulfite solution (pH 5.0) and 24 μL of 10 mM hydroquinone. All solutions were prepared fresh for each analysis. Samples were incubated at 55°C for 16 h. Two drops of mineral oil were layered on top of the solution to prevent evaporation. Bisulfite-modified DNA was purified with the Wizard DNA Clean-up System and vacuum manifold (Promega) according to the manufacturer’s manual. Freshly prepared NaOH solution was added to a final concentration of 0.3 M, and samples were incubated at 37°C for 15 min, followed by neutralization with ammonium acetate (pH 7.0; final concentration 3.0 M) and ethanol precipitation. Normal human peripheral blood lymphocyte DNA samples, treated and untreated with DNA methylase (M. Sss I; New England BioLabs), were also modified as positive and negative controls, respectively.

Methylation-Specific PCR

To assess PTEN promoter methylation in glioma specimens, we used methylation-specific primers as previously described.9 These primers amplified a 181 base-pair region of the PTEN promoter that started 2477 nucleotides upstream from the translation start site. The following primers were used: methylated primers: forward, 5′-GTTTGGGGATTTTTTTTTGC-3′; reverse, 5′-AA CCCTTTCCTACGCCCGG-3′; unmethylated primers: forward, 5′-TATTAGTTGGGATTTTTTTTG-3′; reverse, 5′-CCCACCTTTCCTACCCACA-3′. Aliquots (12 μL) of methylation-specific PCR (MSP) products were analyzed on 3% agarose gels, stained with ethidium bromide, and visualized under UV illumination. Results were recorded with a digital imaging system. For each PCR experiment, DNA samples from peripheral blood samples of normal blood donors treated with and without CpG methylase and bisulfite were included as positive and negative controls, respectively. We repeated MSP assays on all samples and found no discordant results among replicates. The MSP assay is sensitive to approximately 5% methylated product. To confirm the efficiency of the bisulfite modification and the specificity of MSP, bisulfite sequencing of the PCR products was carried out using the procedure reported previously.

Immunohistochemistry

All tissues were routinely fixed in either phosphate buffered 4% formalin or 2% formalin, dehydrated by graded ethanols and embedded in wax (Paraplast Plus, Mc Cormick Scientific) using routine techniques. Five-micron-thick sections were mounted on Superfrost/Plus slides (Fisher Scientific). The following antibodies were obtained from commercial sources and used at the specified dilutions and incubation times/temperatures:
(i) rabbit polyclonal anti-phospho(p)-S6 ribosomal protein (Ser235/236; Cell Signaling #2211) 1:200, 32 min/37℃; (ii) rabbit polyclonal anti-p-S6 ribosomal protein (Ser240/244; Cell Signaling #2215) 1:200, 32 min/37℃; (iii) rabbit monoclonal anti-PTEN (138G6; Cell Signaling #9539) 1:50–1:100, 32 min/37℃; (iv) rabbit polyclonal anti-p-PRAS40 (Invitrogen #441100G) 1:100, 32 min/37℃; and (v) rabbit polyclonal anti-MIB-1 (Ventana Medical Systems Anti-Ki-67 [30-9]) 2 μg/mL/32 min/37℃. Antigen retrieval for p-S6 (both epitopes) and p-PRAS40 was performed for 30 min in Tris buffer pH 8 at 90℃; for PTEN and MIB-1, retrieval time was 16 min. Following antigen retrieval, sections were treated with 3% methanol hydrogen peroxide at 22℃ for 16 min for PTEN and MIB-1 and for 32 min for the p-PRAS40 and p-S6 epitopes. All immunohistochemistry assays were performed on the Ventana Medical Systems Benchmark XT using the Ultraview (multimer) detection system.

All slides were then scored. One score was assigned to each slide based upon the percentage of cells that stained positive. For the percentage staining positive, a score of 0 denoted no positive cells; a score of 1 denoted 1%–33% of cells staining positive; a score of 2 denoted 34%–66% of cells staining positive; and a score of 3 denoted >66% of cells staining positive. Stains were considered positive if a score of 2 or higher was assigned.

Paraffin sections stained with Ki-67 were scanned using a 5× objective of the Leica DMLS microscope fitted with a 25 square graticule. The areas with high numbers of positively stained cells within tumor tissue were located and marked. Using a 20× objective focused on these areas, numbers of positively stained and nonstained cells present within the selected 25 square grid were determined. The procedure was repeated for multiple areas until a total of 1000 cells had been counted. The labeling index percentage was calculated by dividing the number of total positive cells over the number of all cells (positive + negative), and then multiplying by 100. A trained neuropathologist (J.P.) reviewed the quantification.

Statistics

Progression-free survival (PFS) was defined as the time between diagnosis and date of progression or death. Patients without progression were censored at the time of last follow-up. PFS distributions were estimated using the Kaplan–Meier curve, and the log-rank test was used to compare distributions between 2 or more groups. Exact Blyth–Still–Casella confidence intervals were utilized to summarize various proportions, and Fisher’s exact test was used to analyze 2 × 2 contingency tables. For the analyses that involved tables with ordered outcomes both for the rows and the columns, an exact linear-by-linear association test was employed. P-values were not adjusted for multiplicity.

Results

Clinical Characteristics

Table 1 summarizes the clinical characteristics of patients included in this study. A total of 25 grade I, 7 grade II, 7 grade III, and 9 grade IV gliomas were analyzed. Since prior studies reported strong associations between extent of resection and clinical outcome in all grades of pediatric gliomas,10–14 we first investigated the associations between extent of surgical resection and PFS. As shown in Fig. 1A, extent of resection significantly correlated with improved PFS in LGGs (P < .001) but did not reach statistical significance in HGGs (P = .201; Fig. 1B). We also assessed associations between tumor location and PFS. Supratentorial location was associated with worse PFS in children with LGGs (P = .007; Fig. 2A), whereas in HGGs, infratentorial location was associated with inferior outcome (P = .0183; Fig. 2B). These findings are consistent with prior reports in the literature.15,16 Due to the small number in our cohort, we were unable to simultaneously assess associations of extent of resection and location with PFS in a multivariable model.

Immunohistochemical Analyses of PI3K/Akt/mTOR Pathway in Pediatric Gliomas

Immunohistochemistry was used to detect phosphorylated and activated forms of downstream molecules and expression of the PTEN tumor suppressor protein, a critical negative regulator of the PI3K/Akt/mTOR pathway. mTOR is a serine/threonine kinase that consists of 2 multiprotein complexes: mTOR complex (mTORC)1 and mTORC2. The main mTORC1 targets are eukaryotic initiation factor (eIF)4E–binding proteins (4EBP1, 2, and 3) and S6 protein kinases

| Table 1. Clinical characteristics of cohort of pediatric gliomas |
|-----------------|-----------|-----------|-----------|-----------|
| Characteristic   | Grade I   | Grade II  | Grade III | Grade IV  |
| Total number     | 25        | 6         | 7         | 9         |
| Age (y), median (range) | 7.3 (0.1–15) | 6.2 (1.2–15) | 9.8 (0.3–14.7) | 10 (1.2–18) |
| Gender, male:female | 13:12   | 6:1       | 6:1       | 6:3       |
| Number progressed (%) | 8 (32) | 3 (43)     | 3 (43)     | 9 (100)    |
| Follow up (y), mean (range) | 8.9 (0.1–17) | 11.8 (6.1–16.2) | 5.2 (0.8–17.5) | 1.9 (0.3–5.2) |
| Location: supratentorial | 8    | 6         | 5         | 6         |
| Infratentorial   | 17        | 1         | 2         | 3         |
(S6K1 and S6K2). Phosphorylation of 4EBP by mTORC1 releases active elf4E, and phosphorylation of S6K by mTORC1 stimulates S6K activity, with direct consequences of enhanced cap-dependent translation of mRNAs by elf4E and upregulation of overall translational capacity by S6K. Akt phosphorylates PRAS40 and prevents the ability of PRAS40 to suppress mTORC1 signaling. Thus, expression of p-S6, p-4EBP1, and p-PRAS40 reflects activation of the PI3K/Akt/mTOR pathway, although p-4EBP1 is more specific for mTOR activation, since p-S6 can be activated via a variety of cellular mechanisms.

Table 2 summarizes the results of our immunohistochemical analyses by tumor grade. Figure 3A shows a representative example of these immunohistochemical results, and Fig. 3B shows an example of the scoring used in this analysis, based on immunohistochemical staining of p-4EBP1. Twelve out of 15 (80%) high-grade tumors showed activation of the PI3K/Akt/mTOR pathway based on p-S6 status. Expression of p-S6 significantly correlated with p-4EBP1 expression, which was positive in 12/15 (80%) samples. LGG samples demonstrated PI3K/Akt/mTOR pathway activation, as indicated by the presence of p-S6 in 14 out of 32 (43.8%) samples and by the presence of p-4EBP1 in 16 out of 32 (50%) samples. Association tests confirmed that the proportions of p-S6 and p-4EBP1 positivity increased with tumor grade (exact linear-by-linear association test p-S6, \( P = .0075 \); p-4EBP1, \( P = .0066 \)). There was no association between expression of p-S6 and MIB labeling index (data not shown).

The majority of HGG samples were negative for PTEN expression (10 out of 15; 66.7%), whereas the majority of LGGs were positive for PTEN expression (23 out of 32; 71.9%). When we assessed association of tumor grade with PTEN expression, we found that PTEN expression decreased with increasing tumor grade (exact linear-by-linear association test, \( P = .0025 \)). As would be expected from the role of PTEN in regulating PI3K signaling, tumors with PTEN expression had a lower MIB labeling index (Kruskal–Wallis test, \( P = .007 \); Fig. 4).

PTEN Promoter Methylation

PTEN promoter methylation was assessed in samples for which genomic DNA was available \( (n = 39) \). As shown in Table 2, 6 of 17 PAs (35.29%; 95% CI, 16.64%–59.37%) and almost all grade II tumors had PTEN promoter methylation (6 out of 7; 85.7%; 95% CI, 44.58%–99.27%). PTEN promoter methylation was observed in 33% of HGGs (grade III 2/6; 95% CI, 6.285%–72.87% and grade IV 3/9; 95% CI,
We asked whether PTEN promoter methylation was associated with tumor grade and found that PTEN promoter methylation increased with decreasing tumor grade (exact linear-by-linear association test, \( P = .099 \)). We next asked whether PTEN promoter methylation correlated with PTEN protein expression. Of 17 samples with PTEN promoter methylation, 10 had no evidence of PTEN expression. Of 22 samples negative for PTEN promoter methylation, 15 had evidence of PTEN expression, suggesting an inverse association between PTEN promoter methylation and PTEN protein expression (Fisher’s exact test, \( P = .0990 \)).

**PI3K/Akt/mTOR Activation and Clinical Outcome**

We asked whether PI3K/Akt/mTOR pathway activation is associated with clinical outcome in children with gliomas. As shown in Fig. 5, expression of p-S6 and p-4EBP1 showed a trend toward correlation with worse PFS in children with gliomas (p-S6, \( P = .0874 \); p-4EBP1, \( P = .0475 \)). In LGGs, there was a trend suggesting an association of p-4EBP1 with overall survival when stratified by tumor grade (\( P = .0807 \)), although this did not reach statistical significance. Neither

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### Table 2. Immunohistochemical (IHC) analysis of downstream molecules within the PI3K/Akt/mTOR signaling cascade and PTEN promoter methylation status in pediatric gliomas

<table>
<thead>
<tr>
<th>Molecular Marker</th>
<th>Grade I</th>
<th>Grade II</th>
<th>Grade III</th>
<th>Grade IV</th>
</tr>
</thead>
<tbody>
<tr>
<td>PTEN promoter methylation</td>
<td>6/17 (35)</td>
<td>6/7 (86)</td>
<td>2/6 (33)</td>
<td>3/9 (33)</td>
</tr>
<tr>
<td>PTEN expression by IHC</td>
<td>19/25 (76)</td>
<td>4/7 (57)</td>
<td>3/7 (43)</td>
<td>2/8 (25)</td>
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<tr>
<td>Phospho-S6 expression</td>
<td>11/25 (44)</td>
<td>3/7 (43)</td>
<td>5/7 (71)</td>
<td>7/8 (88)</td>
</tr>
<tr>
<td>Phospho-PRAS40</td>
<td>5/25 (20)</td>
<td>1/7 (14)</td>
<td>2/7 (28)</td>
<td>4/4 (100)</td>
</tr>
<tr>
<td>Phospho-4EBP1</td>
<td>12/25 (48)</td>
<td>4/7 (57)</td>
<td>4/7 (57)</td>
<td>8/8 (100)</td>
</tr>
</tbody>
</table>

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Fig. 3. (A) Representative images of tumors that were positive (top row) and negative (bottom row) stained for PTEN, p-PRAS40 (Thr246), p-4EBP1 (Thr37/46), p-S6 (Ser 235/235) and p-S6 (Ser 240/244). PTEN-positive endothelial cells serve as an internal control for PTEN staining. Original magnification 400×. (B) Representative images of sections immunostained for p-4EPB1 demonstrating tumors scored from left to right as 0, 1, 2, and 3. Note there is both cytoplasmic and nuclear immune-positivity. Original magnification 400x.

Fig. 4. MIB-labeling index according to PTEN expression. N, negative PTEN expression; Y, positive PTEN expression (\( P = .007 \)).

9.775%–70.07%). We asked whether PTEN promoter methylation was associated with tumor grade and found that PTEN promoter methylation increased with decreasing tumor grade (exact linear-by-linear association test, \( P = .099 \)).
PTEN promoter methylation nor PTEN expression nor p-PRAS40 correlated with clinical outcome.

**Discussion**

We examined activation of the PI3K/Akt/mTOR pathway, PTEN promoter methylation, and PTEN protein expression in pediatric gliomas of all grades. To our knowledge this is the first study to assess PTEN promoter methylation in pediatric gliomas as an alternate mechanism of PI3K/Akt/mTOR signaling activation. PTEN is a negative regulator of the PI3K/Akt/mTOR pathway, and PTEN mutations lead to constitutive activation of the signaling cascade. Previous studies have shown that childhood gliomas infrequently exhibit amplification of epidermal growth factor receptor or PTEN mutations/deletions. A recent analysis of pediatric gliomas collected by the Children’s Oncology Group revealed that the PI3K/Akt/mTOR pathway is activated in the majority of pediatric HGGs based on phosphorylation of Akt. Akt was activated in 79% (42/53) of pediatric HGGs and 69% of tumors with evidence that Akt activation was associated with loss of PTEN immunoreactivity. However, only a minority of samples showed loss of heterozygosity of chromosome 10, which harbors PTEN. PTEN promoter methylation is an alternate mechanism of PTEN dysregulation and is present in over 80% of secondary adult glioblastoma multiforme (GBM), whereas PTEN deletions are found mainly in primary adult GBM. Since PTEN deletions and mutations are rarely found in pediatric gliomas, we asked whether PTEN promoter methylation is an alternate mechanism that may lead to PI3K/Akt/mTOR pathway activation. Indeed, we found that PTEN promoter methylation is a common event in pediatric gliomas of all grades (44% of investigated cases), significantly correlated with decreased PTEN protein expression, and increased as tumor grade decreased.

Activation of PI3K/Akt/mTOR in adult LGGs portends worse clinical outcome compared to pathway activation, and tumors PI3K/Akt/mTOR are particularly sensitive to mTOR inhibition. Taken together, these findings have important therapeutic implications for LGGs and have formed the rationale for an ongoing phase II clinical trial testing an mTOR inhibitor (everolimus [RAD001]) in adults with recurrent LGGs. We report PI3K/Akt/mTOR activation in the majority of pediatric HGGs and approximately half of LGGs, as reflected by p-S6 and p-4EBP1 expression. In addition, in single-variable models, we observed that expression of p-S6 and p-4EBP1 was associated with worse PFS; however, this association may be dependent on tumor grade rather than serve as an independent marker of clinical outcome. This possible explanation notwithstanding, the association of p-4EBP1 with overall survival in LGGs maintained borderline significance, even when stratified by tumor grade (P = .0807). Due to the retrospective nature of this study and the limited sample size for each tumor grade, we were unable to adjust for known factors that influence clinical outcome, such as extent of resection, treatment, and location. A larger prospective study is needed to answer the question of whether PI3K/Akt/mTOR pathway activation is an independent prognostic factor in pediatric gliomas.

In our current analysis, PFS correlated with extent of resection in the LGG cohort but not in the HGG group. This finding contrasts with studies in which extent of resection correlates with outcome in gliomas of all grades and is likely the result of the limited number of HGG samples. Another limitation is that the relatively small sample size limits our ability to evaluate whether expression levels of p-S6 or p-4EBP1 are associated with extent of tumor resection. In our recent publication of a study of PI3K/Akt/mTOR pathway activation in adult LGGs, we found that expression of p-S6 was still associated with worse clinical outcome than tumors lacking p-S6 expression, even in patients with a subtotal resection.

Fig. 5. Progression-free survival of pediatric gliomas according to (A) phospho-S6 staining (P = .0874) and (B) phospho-4EBP1 staining (P = .0475).
This study demonstrated that PI3K/Akt/mTOR activation occurs in a significant proportion of pediatric LGGs and HGGs. We identified PTEN promoter methylation as an alternate mechanism of PI3K/Akt/mTOR pathway activation in pediatric gliomas. To our knowledge, this is the first description of this alternate mechanism of PI3K/Akt/mTOR activation in pediatric gliomas. Similar to what is observed in adult LGGs, the inverse association between PTEN promoter methylation and PTEN protein expression suggests that PTEN promoter methylation is an alternate mechanism of PTEN inactivation and PI3K/Akt/mTOR activation in pediatric gliomas. These findings provide a strong preclinical rationale to test inhibitors of the PI3K/Akt/mTOR pathway, including mTOR inhibitors and emerging dual PI3K/mTOR inhibitors, in children with gliomas.

Conflict of interest statement. None declared.

Funding

This research was supported in part by the National Institute of Neurological Disorders and Stroke grant K12NS001692 (S.M.), National Institutes of Health Brain Tumor SPORE grant P50 CA097257 (D.H.K., W.A.W., M.S.B.), Nancy and Stephen Grand Philanthropic Fund (D.H.K.), and The V Foundation (S.M., W.A.W., D.H.K.).

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