PI3K Pathway Activation Provides a Novel Therapeutic Target for Pediatric Ependymoma and Is an Independent Marker of Progression-Free Survival

Hazel A. Rogers, Cerys Mayne, Rebecca J. Chapman, John-Paul Kilday, Beth Coyle, and Richard G. Grundy

Abstract

Purpose: Currently, there are few effective adjuvant therapies for pediatric ependymoma outside confocal radiation, and prognosis remains poor. The phosphoinositide 3-kinase (PI3K) pathway is one of the most commonly activated pathways in cancer. PI3Ks transduce signals from growth factors and cytokines, resulting in the phosphorylation and activation of AKT, which in turn induces changes in cell growth, proliferation, and apoptosis.

Experimental Design: PI3K pathway status was analyzed in ependymoma using gene expression data and immunohistochemical analysis of phosphorylated AKT (P-AKT). The effect of the PI3K pathway on cell proliferation was investigated by immunohistochemical analysis of cyclin D1 and Ki67, plus in vitro functional analysis. To identify a potential mechanism of PI3K pathway activation, PTEN protein expression and the mutation status of PI3K catalytic subunit α-isoform gene (PIK3CA) was investigated.

Results: Genes in the pathway displayed significantly higher expression in supratentorial than in posterior fossa and spinal ependymomas. P-AKT protein expression, indicating pathway activation, was seen in 72% of tumors (n = 169) and P-AKT expression was found to be an independent marker of a poorer progression-free survival. A significant association between PI3K pathway activation and cell proliferation was identified, suggesting that pathway activation was influencing this process. PTEN protein loss was not associated with P-AKT staining and no mutations were identified in PIK3CA.

Conclusions: Our results suggest that the PI3K pathway could act as a biomarker, not only identifying patients with a worse prognosis but also those that could be treated with therapies targeted against the pathway, a strategy potentially effective in a high percentage of ependymoma patients. Clin Cancer Res; 19(23); 1–11. ©2013 AACR.

Introduction

Ependymoma is the second most common malignant pediatric tumor of the central nervous system (CNS). Prognosis is relatively poor with a 5-year overall survival (OS) rate of 24% to 82% (1–3). More than half of cases occur in children younger than 5 years (4–6). Ninety percent of pediatric tumors arise intracranially, 70% occurring in the posterior fossa, with the remaining 10% occurring in the spinal cord.

Array-based studies have identified genetic, epigenetic, and transcriptional differences between tumors arising in different anatomic locations (7–9). However, despite the large amount of molecular data generated, few candidate genes and pathways have been identified that could be used for targeted therapies. Several prognostic markers have been identified for ependymoma, but few have been analyzed in sufficient pediatric patients (10). The extent of surgical resection is still the most consistent marker of outcome (11, 12). However, even with complete resection, tumor recurrence occurs in up to 50% of patients (13). A number of putative biologic and genomic prognostic markers have been identified in large cohorts, including nucleolin (14), Ki67 (15), 1q gain (16), nestin (17), Nell2, and Lama2 (18).

The phosphoinositide 3-kinase (PI3K) pathway is one of the most commonly activated pathways in cancer, including glioma and medulloblastoma (19, 20). PI3Ks are lipid kinases that transduce signals from growth factors and cytokines influencing diverse biologic functions including cellular proliferation, survival, and motility. In response to upstream signals, PI3K phosphorylates the lipid phosphatidylinositol-4,5-bisphosphate, converting it to phosphatidylinositol-4,5-bisphosphate and phosphatidylinositol-3,4,5-trisphosphate.
**Translational Relevance**

Brain tumors are the leading cause of cancer-related deaths in children in the United Kingdom. Ependymoma is the second most common malignant pediatric tumor of the central nervous system. Currently, there are few effective adjuvant therapies for pediatric ependymoma outside confocal radiation, and prognosis remains poor. The PI3K pathway is one of the most commonly activated pathways in cancer with a number of drugs targeting the pathway in current clinical trials. Here, we identify the PI3K pathway as a potential therapeutic target and prognostic marker for ependymoma. We found the PI3K pathway to be active in 72% of primary pediatric ependymomas, suggesting that targeting the pathway therapeutically would be a strategy applicable to a high percentage of patients. Through in vitro functional analysis, we demonstrated a significant link between PI3K pathway activation and cell proliferation, suggesting that inhibiting the pathway clinically would reduce ependymoma growth.

3,4,5-trisphosphate (PIP₃; ref. 21). PIP3 links PI3K to downstream effectors including the serine threonine kinase AKT. When the PI3K pathway is activated, AKT becomes phosphorylated and consequently phosphorylates downstream effectors including GSK3β, FOXO transcription factors, and components of the mTOR pathway (22).

In cancer, the PI3K pathway is activated through mutations or alterations in many components of the pathway (23–25). PTEN is a key negative regulator of the pathway and loss of PTEN through mutation or deletion has been reported in many cancers, including glioma (23, 25). Copy-number loss of the PTEN loci has also been seen in ependymoma (7). Activating mutations in the PI3K catalytic subunit α-isoform gene (PIK3CA) are also found in many common cancers (24).

The high incidence of PI3K pathway activation in cancer has made it an attractive therapeutic target. Drugs targeting the PI3K pathway have been developed, many of which are being introduced into clinical trials (22).

We investigated the status of the PI3K pathway in ependymoma. Analysis of mRNA expression array data and protein expression by immunohistochemistry (IHC) demonstrated the PI3K pathway to be active in 72% of primary ependymomas, with a higher percentage of supratentorial ependymomas displaying pathway activation than posterior fossa and spinal tumors. Pathway activation was identified as an independent marker of poorer progression-free survival (PFS). Our data also suggest that PI3K pathway activation induces cell proliferation in ependymoma.

**Materials and Methods**

**Sample information**

Of note, 272 formalin-fixed paraffin-embedded pediatric ependymoma samples were used, 197 primary and 75 recurrent. Primary and subsequent recurrent samples were used from 30 patients. Samples were collected from patients diagnosed between 1961 and 2010. The mean and median age of the cohort was 69 and 46 months, respectively. Sixty-seven percent of primary tumors were posterior fossa, 22% supratentorial, and 6% were spinal. Data were unavailable for nine tumors. Sixty-four percent of primary tumors were classic and 33% were anaplastic ependymoma. Histopathologic information was unavailable for five samples. For patients still alive, the mean time of follow-up was 89 months and median was 83 months. Sixty percent of patients received radiotherapy. Treatment was unknown for 15% of patients.

Retrospective tumor samples were obtained from the Children’s Cancer and Leukaemia Group (CCLG) and the Cooperative Human Tissue Network (CHTN). Approximately 50% of the cohort were samples from patients enrolled in two clinical trials, CNS9204 (26) and CNS9904 (27). Sixty-three primary and 27 recurrent samples were from the CNS9204 trial and 46 primary and 16 recurrent samples were from the CNS9904 trial.

Twenty-three snap-frozen ependymomas were obtained for DNA extraction, 18 primary and 5 recurrent. For 1 patient, the primary and two recurrent samples were included. Samples were collected from patients diagnosed between 1987 and 2007. Fifty-six percent were posterior fossa, 35% supratentorial, and 4% were spinal. For one sample, location was unknown. Sixty-one percent were classic and 39% were anaplastic ependymoma.

Nineteen snap-frozen ependymomas were obtained for RNA extraction, 14 primary and 5 recurrent. Two recurrences were from 1 patient. Samples were collected from patients diagnosed between 1989 and 2008. Sixty-eight percent were posterior fossa, 21% supratentorial, and 11% were spinal. Sixty-three percent were classic and 37% were anaplastic ependymoma. Six of the tumors used for RNA extraction also had DNA extracted.

Patient clinical information was obtained from CCLG, CHTN, local centers, and trial centers. Multiple Centre Research Ethics (MREC) approval was obtained for the study. Consent for use of tumor samples was taken in accordance with national tumor banking procedures and the Human Tissue Act. Work was conducted in premises licensed under the Human Tissue Act.

**Expression array analysis**

Analysis was undertaken using previously published mRNA expression data ( GEO accession number GSE21687; ref. 7). Raw data were processed using the robust multiaarray average (RMA) algorithm. Adult tumors were excluded. Genes differentially expressed between groups (P < 0.05) were identified using an ANOVA test with a Benjamini and Hochberg multiple test correction (28), using GeneSpring GX11.0 software (Agilent). Identified gene lists were analyzed using Ingenuity Pathway Analysis (IPA) 9.0 (http://www.ingenuity.com).
Quantitative PCR
RNA was extracted from 40 to 50 mg of frozen tissue using the mirVana miRNA Isolation Kit (Applied Biosystems). Before extraction, a small piece of tissue was prepared on a glass slide as a diagnostic smear, with subsequent hematoxylin and cosin (H&E) staining, to determine whether tumor cells were present. After extraction, RNA was treated with DNase (Promega; 2 U) at 37°C for 30 minutes. cDNA synthesis was carried out using the RevertAid cDNA Synthesis Kit (Fermentas) using 500 ng RNA. Primers were designed to amplify AKT1 (Forward 5'-CTAAGGGCGCTGTCTCTGAGTGCAG-3', Reverse 5'-GAGGGCTTTCCTGTCACAAAC-3', and annealing temperature 62°C), PIK3R2 (Forward 5'-ATGTTCGTCATGGGTGTTCTTGCTGTAAATTCTAATGCT-3', Reverse 5'-GCTGTAACATGCGAGGTGTCAG-3', and annealing temperature 62°C), FGFR3 (Forward 5'-GCCCTGGACCTGTAACCCCTTGA-3', Reverse 5'-CGTCGCTGGGTTAACAAAAT-3', and annealing temperature 62°C), and GAPDH (Forward 5'-GCCTGGACTGCTACCTTTCA-3', Reverse 5'-CGCTGGACTGCTACCTTTCA-3', and annealing temperature 62°C). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as a housekeeping gene (Forward 5'-ATGTTGGGGGTCCTAACTCC-3', Reverse 5'-ATGTTGGGGGTCCTAACTCC-3', and annealing temperature 62°C). PCR reactions were carried out by Source Bioscience. Primers were designed to amplify exon 9 (forward 5'-CTGTGAATCCAGAGGGGAAA-3' and reverse 5'-CCTTTAGCCCTAATTTTGAC-3') and exon 20 (forward 5'-TTGTCCAGGAGATGTGTTACACG-3' and reverse 5'-TGTTCTTGCTGTAATCTTCTAIGCT-3') of PIK3CA. Standard PCR reactions were carried out. PCR products were purified by incubation with 0.3 U shrimp alkaline phosphatase (Promega) and 1.5 U exonuclease I (NEB) at 37°C for 8 minutes followed by 15 minutes at 72°C. Sequencing reactions were carried out by Source Bioscience.

Cell culture
The ependymoma cell line BXD-1425EPN was provided by Dr. Xiao-Nan Li, Baylor College of Medicine, and has been previously characterized (31). The cell line was derived from a 9-year-old male patient with a recurrent supratentorial anaplastic ependymoma. The EPN1 cell line was generated and characterized in house (32). The cell line was derived from a 13-year-old male patient with a recurrent supratentorial ependymoma. The Res196 cell line was provided by Dr. D.G. van Vuurden, VU University Medical Center, Amsterdam. The line was originally generated and characterized by Bobola and colleagues (33) and was derived from a 4-year-old male patient with a grade 2 ependymoma.

Cells were cultured in standard humidified incubators at 5% CO2. BXD-1425EPN and EPN1 lines were cultured in Dulbecco's Modified Eagle Medium (DMEM; Invitrogen) supplemented with 10% (BXD-1425EPN) or 15% (EPN1) FBS (PAA Laboratories) plus antibiotics. Res196 was cultured in DMEM F12 (Invitrogen) with 10% FBS and antibiotics.

Western blot analysis
Cells were seeded in serum-free medium and incubated overnight. The following day, cells were treated with Ly294002 (Cell Signaling Technology), BKM120 (Adooq Bioscience), or vehicle control (dimethyl sulfoxide, DMSO). After 72 hours, the level of proliferation was measured using the Cell Proliferation Kit I (MTT) following manufacturer’s instructions (Roche). Each condition was performed in triplicate, in three independent experiments.

DNA extraction
DNA was extracted from 5 to 10 mg of frozen tumor tissue as described previously (30). Before tissue extraction, a small piece of tissue was prepared on a glass slide as a diagnostic smear, with subsequent (H&E) staining, to determine whether tumor cells were present.

IHC
Samples were analyzed on tissue microarrays. Following diagnostic pathology review, representative areas of tumor tissue were selected. Three cores from each tumor were included. IHC was carried out as described previously (14). Slides were incubated with phosphorylated AKT (P-AKT; ser 473; 1/50; Cell Signaling Technology), cyclin D1 (1/100; Abcam), phosphorylated S6 (P-S6; 1/100; Cell Signaling Technology), Ki67 (1/50; Dako), and PTEN (1/25; Abcam). P-AKT was scored as positive or negative, with the presence of nuclear staining noted. For cyclin D1, the percentage of positive cells was calculated. At least 100 cells per sample were counted. P-S6 was scored as positive or negative. The Ki67 labeling index was calculated as previously described (14). PTEN was scored as positive or negative.

The association between clinical factors and immunohistochemical status was investigated using the Fisher exact test for categoric variables and t-test for continuous variables. OS and PFS were investigated with the Kaplan–Meier method, with differences estimated using the log-rank (Mantel–Cox) test. OS was defined as the time between date of diagnosis and date of death and PFS as the time between date of diagnosis and date of first event (recurrence/death). Patients still alive at the end of the study were censored at the date of the last follow-up. Multiple factors were analyzed by the Cox proportional hazard regression model to determine OS comparisons with 95% confidence intervals (CI). Variables with a P value equal or less than 0.25 in univariate analysis were included in multivariate analysis.
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(34). Membranes were incubated with primary antibodies to P-AKT (1/1,000; Cell Signaling Technology), AKT (1/2,000; Cell Signaling Technology), and GAPDH (1/50,000; Abcam).

Results

PI3K pathway was associated with tumor location at the gene expression level

Previous research has shown that ependymomas from different locations display distinct biologic profiles. Using published mRNA expression array data (GSE21687; ref. 7), we identified significant differences in mRNA expression between ependymomas from different locations (posterior fossa, supratentorial, and spinal; Supplementary Table S1). Analysis of the top 3,000 probes using IPA identified pathways displaying a significant enrichment in the identified gene list. The top pathway was the PI3K pathway with multiple genes displaying significantly higher expression in supratentorial tumors (Fig. 1A). Seventy-three genes from the PI3K pathway displayed a significant difference in gene expression between ependymomas from different locations (Supplementary Table S2 and Fig. 1B). Genes included components of the pathway such as the AKT genes, growth factors, and their receptors, plus downstream genes including cyclin D1 (CCND1).

Four genes were selected for validation using quantitative PCR: AKT1, PIK3R2, FGFR3, and CCND1. PCR was carried out using an independent cohort of ependymomas to those used in the array analysis. Each gene displayed higher expression in supratentorial ependymomas than in posterior fossa and spinal tumors, agreeing with the array data (Fig. 1C).

PI3K pathway activation was linked to prognosis

AKT is a major downstream effector of the PI3K pathway. The protein expression of P-AKT was used to assess the activation status of the PI3K pathway in a retrospective cohort of 231 ependymomas (169 primary and 62 recurrent). Positive staining was seen in 122 (72%) primary tumors. Of the positive samples, 102 (84%) displayed nuclear staining (P-AKT-N; Fig. 2). A significant association was identified between P-AKT staining and tumor location (P = 0.05), with a higher percentage of supratentorial ependymomas displaying positive staining (84%) than posterior fossa (69%) or spinal tumors (50%). No association was found with any other clinical factor. Fifty (81%) recurrences displayed positive staining, 45 with nuclear staining. Of 24 primary/recurrent pairs analyzed, 19 (79%) displayed positive staining in both tumors. Three were negative in the primary and positive in the recurrent tumor. One pair were both negative and one pair displayed positive staining in the primary tumor that was lost in the recurrent tumor (Supplementary Table S3).

A significant association was identified between P-AKT staining and a worse PFS (P = 0.049). A more significant result was obtained when only the nuclear positive tumors were compared with the rest of the cohort (P = 0.023; Fig. 2).

No association was found with OS. A higher level of significance was seen in multivariate analysis (P-AKT P = 0.02, P-AKT-N P = 0.011; Table 1). Only factors with a P value less than 0.25 in univariate analysis were included in the multivariate analysis. These factors were histology, extent of resection, age, Ki67, location, and whether patients received radiotherapy before progression (Supplementary Table S4). The spinal group was too small to analyze independently. In univariate analysis, the posterior fossa group displayed the most significant association with survival compared with the other two locations. Therefore, supratentorial and spinal tumors were grouped together and compared with posterior fossa tumors for multivariate analysis.

1q status was analyzed in a previous study on a subset of the cohort (n = 76, ~50% of the total cohort; ref. 16). P-AKT was found to be independent of 1q status in a multivariate analysis of the reduced cohort (data not shown). Nucleolin was also analyzed, but did not reach significance in univariate analysis (data not shown).

A recent publication has divided posterior fossa ependymomas into two subgroups that can be identified using Lama2 and nell2 protein expression (18). Lama2 and nell2 protein expression was analyzed in our cohort using IHC. However, no correlation was found with P-AKT; suggesting that PI3K pathway activation was not associated with the posterior fossa subgroups (data not shown).

PI3K pathway activation was associated with cyclin D1 expression and cell proliferation

PI3K pathway activation is known to play a role in control of cell proliferation, including through increased expression of cyclin D1. We found that cyclin D1 gene expression was significantly higher in supratentorial tumors (P = 0.01; Fig. 3). Cyclin D1 expression also significantly correlated with AKT1 expression (r = 0.588; P = 1.6 x 10^-6; n = 64). Cyclin D1 protein expression was investigated in our cohort using IHC. Of note, 140 of 161 (87%) primary tumors and 44 of 50 (88%) recurrences displayed positive staining (Fig. 3 and Supplementary Table S3). A significant difference in the percentage of cyclin D1–positive nuclei was identified between tumors displaying positive or negative P-AKT staining (P = 0.003; n = 144; Fig. 3).

The correlation with cyclin D1 suggested that PI3K pathway activation through AKT may be influencing cell proliferation. We therefore looked at the proliferation marker Ki67 using IHC. The percentage of nuclei displaying positive Ki67 staining ranged from 0% to 65% in primary tumors (Fig. 3). However, Ki67 did not show a significant correlation with P-AKT staining.

To investigate the role of the PI3K pathway in ependymoma functionally, we treated ependymoma cell lines with the PI3K pathway inhibitors Ly294002 and BKM120. Decreased P-AKT protein expression was seen in cells cultured with inhibitor compared with vehicle alone, suggesting that the drugs were specifically inhibiting the PI3K pathway (Fig. 4A). A significant decrease in cell proliferation was seen in drug-treated cells after 72 hours...
Figure 1. Analysis of genes displaying significant differences in mRNA expression between ependymomas from different locations (posterior fossa, supratentorial, and spinal), using IPA, identified pathways significantly enriched. A, the top pathway was the PI3K pathway. Genes highlighted in green were upregulated in supratentorial tumors and genes in pink were upregulated in posterior fossa tumors. The majority of genes displayed higher expression in supratentorial tumors. B, hierarchical clustering of PI3K genes displaying significant differences segregated ependymomas according to location. Analysis was undertaken using previously published expression array data [Johnson and colleagues (7); GSE21687]. The dendogram displayed along the horizontal axis represents the clustering of the ependymoma tumors. The dendogram displayed along the vertical axis represents the clustering of the genes. C, validation of AKT1, PIK3R2, FGFR2, and CCND1 gene expression using quantitative PCR. Each gene displayed higher expression in supratentorial ependymomas than in posterior fossa and spinal tumors, agreeing with the array data. Expression is displayed relative to fetal temporal lobe. PF, posterior fossa; ST, supratentorial; SP, spinal.
IC₅₀ concentrations for Ly294002 were 40, 20, and 50 μmol/L for BXD-1425EPN, Res196, and EPN1 cell lines, respectively. IC₅₀ concentrations for BKM120 were 2.7, 1.3, and 4.4 μmol/L for BXD-1425EPN, Res196, and EPN1 cell lines, respectively. These results suggest that the PI3K pathway is increasing cell proliferation in ependymoma.

PI3K pathway activation was not linked to mTOR pathway activation

Activation of the PI3K pathway can induce activation of the mTOR pathway. The mTOR pathway can also directly regulate the PI3K pathway. mTOR pathway status was investigated in our tissue microarray (TMA) cohort using P-S6 to indicate pathway activation. Sixty-six of 146 (45%) AKT-negative samples were positive for P-S6, and 107 of 146 (73%) AKT-cytoplasmic samples were positive. In univariate survival analysis, a significant association with a worse PFS was found for both P-AKT and P-AKT-N.

![Figure 2](image_url)

**Figure 2.** Three patterns of P-AKT protein expression were seen in ependymoma, measured by IHC, negative (A), cytoplasmic (B), and nuclear (C). D, in univariate survival analysis, a significant association with a worse PFS was found for both P-AKT and P-AKT-N.

Table 1. Multivariate survival analysis

<table>
<thead>
<tr>
<th>Factor</th>
<th>Significance</th>
<th>HR (95% CI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>P-AKT (positive vs. negative)</td>
<td>0.020*</td>
<td>2.060 (1.120–3.788)</td>
</tr>
<tr>
<td>Histology (ependymoma vs. anaplastic ependymoma)</td>
<td>0.617</td>
<td>0.873 (0.512–1.487)</td>
</tr>
<tr>
<td>Resection (incomplete vs. complete)</td>
<td>3.0 × 10⁻⁴ᵇ</td>
<td>2.65 (1.565–4.487)</td>
</tr>
<tr>
<td>Location (PF vs. ST+SP)</td>
<td>0.088</td>
<td>1.784 (0.917–3.470)</td>
</tr>
<tr>
<td>Age (continuous variable)</td>
<td>0.540</td>
<td>1.002 (0.995–1.009)</td>
</tr>
<tr>
<td>Ki67 (continuous variable)</td>
<td>0.111</td>
<td>1.061 (0.986–1.142)</td>
</tr>
<tr>
<td>Radiotherapy (yes vs. no)</td>
<td>0.129</td>
<td>0.618 (0.332–1.150)</td>
</tr>
</tbody>
</table>

| P-AKT-N (nuclear vs. cytoplasmic or negative) | 0.011*       | 2.012 (1.179–3.456) |
| Histology (ependymoma vs. anaplastic ependymoma) | 0.546       | 0.849 (0.501–1.442) |
| Resection (incomplete vs. complete) | 0.001ᵇ       | 2.518 (1.494–4.243) |
| Location (PF vs. ST+SP)             | 0.099        | 1.744 (0.901–3.373) |
| Age (continuous variable)           | 0.628        | 1.002 (0.995–1.009) |
| Ki67 (continuous variable)          | 0.078        | 1.067 (0.993–1.147) |
| Radiotherapy (yes vs. no)           | 0.176        | 0.652 (0.351–1.212) |

Abbreviations: PF, posterior fossa; ST, supratentorial; SP, spinal.

*P < 0.05, ᵃᵇP < 0.01.
primary and 23 of 52 (44%) recurrent tumors displayed positive staining (Fig. 3 and Supplementary Table S3). No correlation was found between P-AKT and P-S6 staining, suggesting that the two pathways were not linked in ependymoma. No association between P-S6 and OS or PFS was identified.

PI3K pathway activation was not caused by PTEN loss or PIK3CA mutations

PTEN DNA copy-number loss has previously been reported in ependymoma, which correlated with gene expression data (7), suggesting that this could be the mechanism of PI3K pathway activation. We investigated PTEN protein levels using IHC. Ten of 142 (7%) primary tumors and 2 of 49 (4%) recurrences were negative (Fig. 5 and Supplementary Table S3). PTEN protein expression was not associated with P-AKT status, suggesting that PTEN loss was not causing PI3K pathway activation.

The presence of activating mutations in PI3KCA was also investigated. Exons 9 and 20 (mutation hotspots) were sequenced in DNA extracted from 23 ependymomas. No mutations were found.

Discussion

We have identified PI3K pathway activation in 72% primary pediatric ependymomas, suggesting that the pathway is a strong candidate for targeted therapy, likely to be effective in a high proportion of patients. Our results have also shown pathway status to be an independent marker of poor prognosis and suggested that pathway activation was playing a role in the control of cell proliferation, which may be acting through cyclin D1.

The results from this study suggest that inhibiting the PI3K pathway therapeutically in pediatric ependymoma is an attractive option and is applicable to a high percentage of patients. In a recent drug screen of a model of a supratentorial subtype of ependymoma, inhibitors of IGF1R, which signals through the PI3K pathway, and GSK3β were found to disrupt cell proliferation, supporting our conclusions (35). A number of drugs have been developed against the PI3K pathway, many of which are undergoing clinical trials, including in adult glioma (22, 36). The IC50 concentrations for BKM120 that we found for ependymoma were similar to those seen in other types of cancers, including adult glioma (37). Phase I studies in adult tumors have demonstrated that effective plasma concentrations of BKM120 can be achieved in patients (38). However, phase I studies of BKM120 have not been conducted in pediatric patients.

We found PI3K pathway status, defined using P-AKT, to be an independent prognostic marker of a poorer PFS in ependymoma, in comparison with clinical factors and previously reported markers. The PI3K pathway has also been linked to a poorer prognosis in other cancers including glioma and breast cancer (19, 39).

Our analysis was undertaken in a retrospective cohort, which included infants and older children and a variety of treatments. Age at diagnosis and treatment, including extent...
of resection and radiotherapy, have previously been linked to prognosis (11, 12, 40–43). The multivariate survival analysis that we undertook demonstrated that the association of P-AKT with PFS was independent of these confounding factors. However, to confirm our result, pathway status must be investigated in clinical trial cohorts. Our cohort contained a subset from patients enrolled on two clinical trials, the CNS9204 and CNS9904 trials. When these were analyzed independently, a significant association with PFS was only found for P-AKT-N in the CNS9204 trial (Supplementary Fig. 1). However, P values were close to significance in all analyses and suggested the same trend for a worse PFS in patients with tumors displaying PI3K pathway activation. It is likely that significance was lost due to the lower number of tumors analyzed in each cohort.

The association with PFS was more significant when tumors displaying nuclear positivity only were compared with the rest of the cohort. The cellular localization of AKT can determine the downstream effects of activation (44). Nuclear specific functions for AKT in tumor development and invasion have been demonstrated in cancer models (45, 46).

If PI3K pathway status is confirmed as a prognostic marker in independent ependymoma cohorts, it could be considered as an attractive marker as it would not only identify patients with a poorer prognosis but also those that could be treated with therapies targeted against the pathway.

Our analysis suggested that PI3K pathway activation in ependymoma was influencing cell proliferation. PI3K pathway activation has been linked to cell proliferation in other cancers including medulloblastoma and glioma (20, 47). Our results suggest that the PI3K pathway may be influencing cell proliferation through cyclin D1. The PI3K pathway has been shown to control cell proliferation through a number of mechanisms, including regulation of cyclin D1 levels through GSK3β (48, 49). Two previous studies analyzing cyclin D1 in ependymoma found a lower percentage of positive tumors than we identified (50, 51). However, both studies analyzed a relatively low number of patients and included a high percentage of adult tumors. A third study analyzed pediatric cases only with results closer to our findings (52).

P-AKT expression did not correlate with the proliferation marker Ki67, which might suggest that the change in cyclin D1 levels was not affecting cell proliferation. However, Ki67 is a universal marker of proliferating cells that can be influenced by many mechanisms that control cell division and is not directly related to cyclin D1 expression. It may be that cell proliferation is being controlled by different mechanisms in ependymomas lacking PI3K pathway activation, hence masking any effect the PI3K pathway may be having. In breast cancer, a positive correlation was seen between P-AKT and cyclin D1 without correlation with Ki67, similar to our results (39). The reduction in cell proliferation that we saw upon PI3K pathway inhibition in the ependymoma cell lines

Figure 4. Ependymoma cell lines were treated with indicated concentrations of Ly294002 and BKM120 for 1 hour, with EGF (50 ng/mL) stimulation for the last 10 minutes. Loss of P-AKT was seen with increasing concentrations of each inhibitor (A). Treatment with the inhibitors resulted in a decrease in cell growth. Cells were incubated with indicated concentrations of the two inhibitors for 72 hours. Cell growth was measured using an MTT assay. A significant decrease in proliferation was seen in drug-treated cells compared with vehıcle control (B). *, P < 0.05; **, P < 0.01.
supports our hypothesis that PI3K pathway activation is increasing cell proliferation in ependymoma.

A significant association between PI3K pathway activation and tumor location was identified at the gene and protein expression levels. This supports previous research that has identified genomic, epigenetic, and transcriptomic differences between ependymomas arising in different locations (7–9). However, a relatively high percentage of posterior fossa and spinal ependymomas displayed PI3K pathway activation, suggesting that it also plays an important role in a subset of these tumors. Supratentorial ependymomas have been associated with a better prognosis than infratentorial tumors, including in the cohort analyzed in this study. This may seem to contradict the association of PI3K pathway activation with both supratentorial ependymomas and a poorer PFS. However, our multivariate survival analysis suggested that the association of PI3K pathway activation with PFS was independent of location.

The PI3K pathway can induce activation of the mTOR pathway, which is also deregulated in cancer, with drugs targeting the pathway in clinical trials for several malignancies (62, 63). We did not find a correlation between PI3K and mTOR pathway status in ependymoma, suggesting that the two pathways were not linked. However, we saw mTOR pathway activation in approximately 50% of ependymomas, suggesting that it may be playing an important role in a proportion of tumors. Activation of one or both of the PI3K and mTOR pathways was seen in 88% of primary ependymoma cases that we analyzed, suggesting that the majority of pediatric ependymoma patients could be treated with therapies targeting one of the pathways.

In conclusion, we have demonstrated the PI3K pathway to be active in a high percentage of ependymomas and to be an independent prognostic marker of a worse PFS. Our data suggest that pathway activation in ependymoma increases cell proliferation. The high percentage of patients with pathway activation and the link to a poor prognosis suggests that the potential of targeting the pathway therapeutically should be investigated. If this proves to be a successful approach, it would be applicable in a high proportion of patients.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

Authors’ Contributions
Conception and design: H.A. Rogers, R.G. Grundy
Development of methodology: H.A. Rogers
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): H.A. Rogers, C. Mayne, R.J. Chapman

Figure 5. PTEN protein expression was measured using IHC. Loss of expression (A) was seen in 7% of primary tumors and positive expression (B) was seen in the remaining 93%.
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): H.A. Rogers, B. Coyle, R.G. Grundy

Writing, review, and/or revision of the manuscript: H.A. Rogers, B. Coyle, R.G. Grundy

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): C. Mayne, R.J. Chapman, J.-P. Kilday

Study supervision: H.A. Rogers, B. Coyle, R.G. Grundy

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