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Akt activation is a common event in pediatric malignant gliomas and a potential adverse prognostic marker: a report from the children's oncology group

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Abstract

Aberrant activation of Akt is a common finding in adult malignant gliomas, resulting in most cases from mutations or deletions involving *PTEN*, which allows constitutive Akt phosphorylation. In contrast, we have previously reported that pediatric malignant gliomas, which are morphologically similar to lesions arising in adults, have a substantially lower incidence of genomic alterations of *PTEN*. The objective of this study was to determine whether Akt activation was also an uncommon finding in childhood malignant gliomas and whether this feature was associated with survival. To address this issue, we examined the frequency of Akt activation, determined by overexpression of

the activated phosphorylated form of Akt (Ser⁴⁷³) on immunohistochemical analysis, in a series of 53 childhood malignant gliomas obtained from newly diagnosed patients treated on the Children's Oncology Group ACNS0126 and 0423 studies. The relationship between Akt activation and p53 over-expression, MIB1 labeling, and tumor histology was evaluated. The association between Akt activation and survival was also assessed. Overexpression of activated Akt was observed in 42 of 53 tumors, far in excess of the frequency of PTEN mutations we have previously observed. There was no association between Akt activation and either histology, p53 overexpression, or MIB1 proliferation indices. Although tumors that lacked Akt overexpression had a trend toward more favorable event-free survival and overall survival ($p = 0.06$), this association reflected that non-overexpressing tumors were significantly more likely to have undergone extensive tumor removal, which was independently associated with outcome. Activation of Akt is a common finding in pediatric malignant gliomas, although it remains uncertain whether this is an independent adverse prognostic factor. In view of the frequency of Akt activation, the evaluation of molecularly targeted therapies that inhibit this pathway warrants consideration for these tumors.

Keywords

Anaplastic glioma; Childhood; Glioblastoma; Akt; Prognostic factors; Treatment resistance

Introduction

Brain tumors are collectively the leading cause of childhood cancer-related mortality. High-grade gliomas have a particularly poor prognosis, with a long-term survival rate of less than 20%, despite conventional surgical, radio-therapeutic, and chemotherapeutic interventions [1]. Although these tumors are comparable morphologically to malignant gliomas that arise in adults, childhood gliomas have a number of distinctive molecular characteristics, which suggests the involvement of somewhat different pathways of tumorigenesis. For example, childhood primary malignant gliomas rarely exhibit *EGFR* amplification, which is a common feature of adult primary malignant gliomas, but often demonstrate overexpression of the EGFR protein [2–9], presumably mediated by distinct mechanisms. Conversely, pediatric lesions commonly exhibit p53 mutations, similar to adult glioblastomas that progress secondarily from lower grade lesions [10–16]. In addition, previous studies from our group [2] and others [3,5,6] have shown that childhood malignant gliomas infrequently exhibit *PTEN* mutations, another of the hallmarks of adult primary glioblastomas [17], which leads to constitutive activation of Akt (protein kinase B), although these lesions may exhibit activation of Akt by mechanisms other than genomic *PTEN* alterations [8,18].

Given the recent development of molecularly targeted agents that inhibit the aberrantly activated signaling pathways of various tumors, the elucidation of the molecular alterations involved in tumorigenesis is relevant not only from a biological perspective, but also from a therapeutic standpoint. Akt is a serine/threonine kinase that is of particular interest in this regard, owing to its central role as a regulator of cell growth, migration, and survival [19,20], and the availability of several novel agents that inhibit Akt [21], its upstream activator, phosphatidylinositol 3-kinase (PI3 K) [22–28], or components of its signaling network [29–32]. We therefore sought to define the frequency of Akt activation in a centrally reviewed cohort of childhood malignant gliomas, and to determine whether this factor was associated with p53 expression status and MIB1 proliferation index, and whether Akt activation status had an impact on overall survival following conventional multimodality therapy.

Methods

Tumor samples

Tumor samples were obtained from children enrolled on the Children's Oncology Group ACNS0126 and 0423 studies, both of which involved post-surgical administration of alkylator-based therapy and irradiation. Each study required institutional IRB approval for protocol enrollment. Children on ACNS0126 received temozolomide daily with radiation and on a 5-day per 28-day cycle post-irradiation; and those on ACNS0423 received daily temozolomide during irradiation followed by post-irradiation temozolomide and lomustine. Eligibility for both studies required an institutional histopathology diagnosis of high-grade glioma (i.e., glioblastoma (GBM), anaplastic astrocytoma (AA), or other eligible malignant glioma, such as gliosarcoma), and each incorporated central pathology review to refine classification.

Tissue accrual for the current study was coordinated by the Pediatric Branch of the Cooperative Human Tissue Network (CHTN). Specimens were de-identified by the CHTN to mask clinical and outcome data from investigators.

Immunohistochemistry

Paraffin-embedded specimens were used for all analyses. Slides were reviewed by a neuropathologist (RLH) to confirm that tumor tissue of sufficient quantity was available for the planned studies. Tumor-containing sections were baked at 60°C for 30 min, deparaffinized in xylene, and rehydrated in graded concentrations of ethanol. Endogenous peroxidase activity was quenched by incubation in 0.3% hydrogen peroxide solution. Antigen retrieval [33] was performed by heating the slides in 10 mmol citrate buffer (pH 6.0) for 20 min. Nonspecific antibody binding was blocked by incubation in Protein Blocking Reagent (Thermo Corp, Pittsburgh, PA) for 20 min. Sections were then incubated with primary antibodies against p-Akt (Se⁴⁷³) (Cell Signaling Technology, Inc., Danvers, MA; 1:100), Ki-67 (Immunotech, Westbrook, ME; 1:100), and p53 (DO-7, Dako Corporation, Carpinteria, CA; 1:300), in Common Antibody Diluent (BioGenex, San Ramon, CA) at room temperature for 2 h. The p53 antibody recognizes a denaturation-stable determinant of wild-type and mutant p53 [34]. Negative control sections were treated with diluent and mouse IgG (5 µg/ml, Dako Corporation, Carpinteria, CA) alone. Slides were then rinsed twice with PBS and antibody binding was localized using a Universal Labeled Streptavidin–Biotin 2 System (LSAB 2—HRP, Dako). Slides were incubated for 30 min in Biotinylated Link reagent at room temperature, followed by a 10 min PBS wash. Slides were then incubated in Streptavidin-HRP solution for 30 min at room temperature. Antibody binding was visualized using 3,3'-diaminobenzidine [35]. The slides were counterstained with Mayer's hematoxylin, dehydrated through graded concentrations of ethanol, cleared in xylene, mounted and examined using a light microscope. Positive controls were included with each batch of sections to confirm the consistency of the analysis. Where sufficient slides were available, staining for PTEN was also performed, using an primary antibody against wild-type PTEN (Clone 138G6, Cell Signaling. 1:100).

Immunohistochemical analysis

Immunoreactivity of tumor specimens for pAKT was assessed semiquantitatively by a neuropathologist (RLH), and in accordance with other reports, tumors were considered positive for pAkt expression if dense staining at an intensity equal to or greater than that of the positive control was seen in at least 20% of cells [36,37]. Reliability of categorization was subsequently confirmed by blinded independent review of the specimens by a second neuropathologist (GHM). Percent MIB-1 labeling was quantitated as previously reported by our group by counting stained and unstained cells in 5–10 high-power fields (approximately 2,000 cells) that incorporated the most anaplastic regions of the specimen [38]. P53 labeling was assessed semiquantitatively as previously reported [7]. Tumors with more than 10% positive cells, either

focally or diffusely within the lesion, were categorized as exhibiting overexpression in relation to normal brain. PTEN staining was considered deficient if absent in at least 25% of the tumor-containing section and present in adjacent endothelial cells or normal tissue elements.

PTEN LOH Assay

Because previous studies from our group [2] and others [5,6] have shown the mutations of *PTEN* are extremely uncommon in pediatric malignant gliomas, but that loss of heterozygosity (LOH) involving the region of the *PTEN* locus on chromosome 10q is a substantially more frequent event [2], we examined the relationship between Akt immunoreactivity and 10q LOH. Formalin-fixed paraffin-embedded (FFPE) tissue specimens were used for all analyses. Tumor targets were manually microdissected from 4- μ m unstained histologic sections under the guidance of a hematoxylin and eosin-stained slide using an Olympus SZ61 stereo microscope (Olympus, Hamburg, Germany). DNA was isolated from each target with the DNeasy Blood and Tissue kit on the automated QIAcube (Qiagen, Valencia, CA) instrument according to the manufacturer's instructions. The quantity of isolated DNA was assessed using a NanoDrop 1000 spectrophotometer (Thermo Scientific, Wilmington, DE). Detection of loss of heterozygosity involving chromosome 10q was performed using a series of microsatellite markers (D10S520, D10S1171, D10S1173). PCR amplification was performed with primers labeled either with TET, NED or HEX fluorophores at the 5' end and carried out on a 9700 GeneAmp thermocycler (Applied Biosystems, Foster City, CA) using standard PCR profiles (denaturation at 94°C for 10 min followed by 35 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 30 s and extension at 72°C for 1 min). Post-PCR amplification products were detected using capillary gel electrophoresis on an ABI 3730 platform (Applied Biosystems). The relative fluorescence values (peak heights) were analyzed using GeneScan 3.7 software (Applied Biosystems), with a 1.5-fold or greater difference in peak height ratios indicative of LOH.

Central pathology review

Both ACNS0126 and 0423 used a prospective central review panel to restrict the eligible cohort to those cases with high-grade gliomas and refine histological classification according to consistent guidelines [39]. The immunohistochemical analysis was performed independently of this review, so the review diagnosis of each specimen was not known during the assessment.

Statistical analysis

For outcome analysis, tumors were classified according to the presence or absence of Akt activation. The endpoints for analysis were overall survival (OS) and event-free survival (EFS). Overall survival was defined as the time from study entry to death from any cause. Event-free survival was defined as the time from study enrollment to the first occurrence of disease progression, relapse, second malignancy, or death from any cause. Nonparametric EFS and OS curves were computed using the product-limit estimates, with standard error via the Greenwood formula [40]. Comparisons of outcome were based on one-sided log-rank test. Comparisons of the distribution of Akt activation status between different patient subgroups defined by age, gender, histology, extent of resection, p53 expression, and MIB1 labeling were based on two-sided Fisher exact test [41].

Results

Frequency of Akt activation

Fifty-three patients had specimens that were available for analysis of Akt activation and both p53 and MIB1 labeling, with 48 patients from ACNS0423 and 5 from ACNS0126. There were 106 and 90 eligible patients enrolled on ACNS0423 and ACNS0126, respectively. As a group,

the 53 patients who had specimens had a more favorable outcome than the cohort without specimens. Of the 53 evaluable tumors, 11 showed little or no pAkt immunoreactivity, whereas 42 (79%) showed overexpression, with dense staining in more than 20 percent of cells (Fig. 1). The typical pattern of pAkt staining included both cytoplasmic and nuclear immunoreactivity. This is consistent with most other reports for malignant cells regarding the subcellular localization of pAkt staining [36,37,42,43].

The distribution of pAkt immunoreactivity as a function of age, gender, histology, extent of resection, p53 expression status, and MIB1 labeling is shown in Table 1. Among the 42 tumors with Akt activation, 22 (52.4%) also showed p53 overexpression. Among the 11 tumors lacking Akt activation, p53 overexpression was seen in 6 (54.5%). Average MIB labeling in the Akt activated subset was 31.8 ± 3.2 versus 24.7 ± 3.8 in those without pAkt expression ($p = 0.28$). The frequency of particularly high MIB1 indices ($>36\%$), which has been previously associated with an adverse prognosis, did not differ significantly between the two groups (2 of 11 vs. 15 of 42, $p = 0.30$).

We also examined whether Akt activation might be more common in grade IV tumors compared to grade III lesions, given that *PTEN* mutations in adult malignant gliomas are seen predominantly in GBMs. Contrary to this assumption, Akt activation was seen at relatively comparable frequencies in the two histological subgroups, being present in 20 of 24 grade III gliomas (83.3%) and 22 of 29 grade IV lesions (68.9%) ($p = 0.74$). However, a significantly higher percentage of patients with no pAkt overexpression had $\geq 90\%$ extent of resection (81.8%) compared to 40% in the pAkt overexpressing group ($p = 0.02$).

Akt activation and outcome

To assess the prognostic significance of Akt activation in pediatric malignant gliomas, overall survival and event-free survival were compared between the subgroup of tumors showing overexpression of pAkt and those lacking this feature. One-year EFS was $59 \pm 8\%$ for patients with Akt overexpression and $91 \pm 8\%$ for those with no overexpression ($p = 0.16$, logrank test) (Fig. 2). One-year OS was $78 \pm 7\%$ for patients with Akt overexpression versus 100% for those with no overexpression ($p = 0.06$, logrank test) (Fig. 3). The median follow up for non-failures was 2.1 years. Based on the figures presented, it appeared that patients who had tumors with Akt overexpression seemed to fail earlier than those lacking this feature, although more mature follow up data is needed for the comparison of long-term outcome. Given the known association of extent of resection with outcome, we also examined the association between pAkt expression and outcome, after stratifying for extent of resection. After this stratification, some of the early difference in outcome as a function of pAkt status disappeared, suggesting that this difference may in part reflect differences in outcome due to the impact of resection extent. Figures 4 and 5 show the EFS and OS in relation to Akt expression level in patients who had $\geq 90\%$ extent of resection. Corresponding figures in patients with $<90\%$ resection are not shown due to small patient numbers.

Reliability of pAkt categorization

We assessed the reliability of Akt categorization in these specimens by having the immunohistochemical results reviewed independently by a second neuropathologist in a blinded fashion. Concordance among the two reviewers in categorization of Akt immunoreactivity was extremely high, with complete agreement in 51 of 53 cases (96.2%). The two discrepancies related to cases judged to have overexpression by the initial reviewer in which the second reviewer felt the 20% threshold for pAkt immunoreactivity was not reached throughout the tumor specimen.

Association of pAkt overexpression with PTEN immunoreactivity and LOH

Given the known impact of PTEN deletion or inactivation on Akt activation status, we examined whether pAkt overexpression was associated with either LOH of chromosome 10q in the region of the *PTEN* locus or loss of expression of PTEN. Because this was not one of the initial objectives of the study, availability of tissue sections for these analyses was a limiting factor in many instances. LOH studies were performed in 30 of the samples, 26 of which had pAkt overexpression and four of which did not. Seven of the 26 pAkt-overexpressing tumors had chromosome 10q LOH, although in two of these cases, only one of three loci examined showed this alteration. In contrast, 19 had no evidence of LOH. Two of four tumors without Akt overexpression had 10q LOH. Thus, no consistent association between pAkt immunoreactivity and 10q LOH was apparent (two-tail Fisher's exact test *p* value: 0.56). Likewise, in the 15 cases amenable to PTEN immunohistochemistry, 9 of 13 with pAkt overexpression showed loss of PTEN immunoreactivity, whereas 1 of 2 without pAkt overexpression showed loss of PTEN (two-tail Fisher's exact test *p* value: 1.0).

Discussion

Activation of the PI3K/Akt pathway is a common feature of malignant gliomas that arise in adults [16]. Mechanistically, this occurs most commonly as a consequence of mutations of *PTEN*, a tumor suppressor gene that normally regulates downstream inhibition of Akt phosphorylation. Loss of this suppressive signal leads to constitutive Akt activation. Activated Akt phosphorylates several proteins involved in cell survival and cell growth signaling, such as Bad, forkhead transcription factor, glycogen synthase kinase, and the mammalian target of rapamycin (mTOR) [19,20], which promotes tumor cell proliferation, survival and invasion. Although previous studies from our laboratory [2] and others [3,5,6] have shown that *PTEN* mutations are rare in pediatric malignant gliomas, it has been demonstrated that other factors can contribute to dysregulation of the Akt pathway. These include activating mutations of the *PIK3CA* and *PIK3R1* genes [16,44,45], methylation-mediated inactivation of *PTEN* [46] and constitutive activation of upstream signaling elements, such as growth factor receptors [16]. Because Akt is a focus of interest for molecularly targeted therapies in gliomas, we questioned whether Akt activation was a frequent finding in pediatric malignant gliomas.

The results of this study demonstrate that high levels of Akt phosphorylation are commonly observed in pediatric malignant gliomas. It is important to note that phosphorylation of the Ser473 site represents only one surrogate for Akt activation, in addition to phosphorylation of the Thr308 site and activation of downstream signaling elements, such as S6 kinase and S6, among others [36,37,42,47–50]. The Ser473 site has been most commonly profiled in previous studies as a marker of Akt activation status, owing to the reasonably robust immunoreactivity of this antigen in formalin-fixed, paraffin-embedded tissue, which provided a rationale for its use as a marker for Akt activation in the current study.

The high frequency of Akt phosphorylation in this centrally reviewed cohort of tumors is consistent with results from smaller institutional cohorts. For example, Faury et al. [18] noted phosphorylation of Akt in 10 of 18 pediatric malignant gliomas analyzed by western blotting, and 10 of 17 institutional samples examined by immunohistochemistry. They also observed this feature in 13 of 21 samples from an independent data set [18] and noted an adverse association between Akt activation and outcome. Similarly, Thorarinsdottir et al. [8] noted Akt phosphorylation in more than 80% of independent tissue samples derived from 22 pediatric malignant gliomas. High expression of pAkt was associated with high levels of EGFR expression in these tumors.

The association between a more adverse prognosis in tumors with Akt phosphorylation than those lacking this feature fits with observations in a variety of tumor types [36,37,42,43,47–

50]. In part, this may reflect that Akt activation represents a common event in the evolution toward treatment-resistant biological behavior. Because Akt activation is involved in mediating apoptosis resistance, this change may render tumor cells more resistant to the cytotoxic effects of irradiation and conventional chemotherapy. Our observation fits with the findings of Faury et al. [18] that those patients whose tumors had a “Ras active” phenotype, which generally encompassed Akt activation, had a trend toward worse overall survival than those with tumors lacking this feature.

Because previous studies of primary adult malignant gliomas have indicated that *PTEN* alterations are seen most commonly in grade IV gliomas, and infrequently in grade III lesions [51], we examined whether the frequency of Akt activation was associated with tumor histology or other biological factors, such as p53 overexpression and high MIB1 proliferation indices, which have been noted to be more common in grade IV than grade III lesions. Although the latter trend was apparent in this cohort as well, our analysis showed no clear association between tumor grade and the frequency of Akt activation, which was detected in the majority of both grade III and grade IV malignant gliomas. This finding is consistent with the observation of Wiencke et al. [46] in adult secondary malignant gliomas, which exhibit Akt activation at similarly high frequencies in both grade III and grade IV lesions. Secondary adult malignant gliomas resemble pediatric malignant gliomas in terms of their frequency of p53 mutations and infrequency of *PTEN* mutations and *EGFR* amplification, although exhibit key biological differences in terms of their progression from lower grade (i.e. grade II) histologies, which is an uncommon pattern in childhood lesions. It has been suggested that the predominant mechanism of Akt activation in these tumors is *PTEN* inactivation by promoter methylation, rather than mutation [46], and in this setting, it represents an earlier step in malignant progression. Such a mechanism remains to be established in the pediatric glioma context, although it has been shown that a significant percentage of childhood malignant gliomas have loss of PTEN immunoreactivity [8,9], despite their low incidence of *PTEN* mutations. In agreement with these previous results, we also observed that among 15 tumors amenable to PTEN immunohistochemistry, 9 of 13 (69%) with pAkt overexpression exhibited loss of PTEN immunoreactivity, although the molecular basis for this observation remains conjectural. LOH of chromosome 10q was noted in 27% of evaluable tumors with pAkt overexpression, which suggests that other mechanisms, such as *PTEN* methylation, may play a contributory role in at least a subset of these tumors.

An unexpected observation of this study was that the subset of tumors that lacked pAkt overexpression were more likely to have undergone extensive ($\geq 90\%$) tumor resections than those with pAkt overexpression. This may reflect distinctive features in the growth properties of these tumors or their location of origin. However, there was no apparent difference in the frequency of pAkt overexpression in tumor subsets defined by superficial (e.g. cerebral or cerebellar cortical) versus deep (e.g., basal ganglion, diencephalic, or brainstem) localization, so the impact of location as a potential correlative factor remains conjectural. Another potentially important observation was that the frequency of positive and negative pAkt immunoreactivity varied significantly between the ACNS0126 and ACNS0423 study samples. This may reflect true differences between these cohorts in the biology of the evaluable tumors or differences in staining of the pAkt antigen as a function of specimen age, given that the ACNS0126 samples were obtained several years prior to the ACNS0423 samples.

Notwithstanding the uncertainty regarding independent prognostic relevance of pAkt overexpression, the finding of high levels of Akt activation in a sizeable percentage of pediatric malignant gliomas has important implications from a therapeutic perspective. With the availability of molecularly targeted agents that directly inhibit Akt phosphorylation [21] or that interfere with signaling elements upstream or downstream from Akt, such as PI3 K and mTOR [22–32], it may be possible to counteract the growth and survival promoting effects of Akt,

and such tumors may be particularly sensitive to these inhibitors [52,53]. These agents are already being examined in a number of solid tumors, including adult malignant gliomas [26, 29–31], and the results of this study provide a rationale to further explore the potential applicability of this strategy in pediatric malignant gliomas.

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Fig. 1.
Pediatric malignant gliomas exhibiting positive (*left*) and negative (*right*) staining for pAkt

Fig. 2.
Event-free survival in pediatric malignant gliomas exhibiting positive and negative staining for pAkt

Fig. 3.
Overall survival in pediatric malignant gliomas exhibiting positive and negative staining for pAkt

Fig. 4. Event-free survival in patients with 90% extent of resection exhibiting positive and negative staining for pAkt

Fig. 5. Overall survival in patients with 90% extent of resection exhibiting positive and negative staining for pAkt

Table 1

Association Between pAkt overexpression and histology, p53 overexpression and MIB1 labeling

	pAkt overexpression <i>N</i> (%)	No pAkt overexpression <i>N</i> (%)	<i>p</i> value
Study			0.005
ACNS0423	41 (97.6)	7 (63.6)	
ACNS0126	1 (2.4)	4 (36.4)	
Age			0.26
<10 years	10 (23.8)	5 (45.5)	
≥10 years	32 (76.2)	6 (54.5)	
Gender			1.0
Male	25 (59.5)	6 (54.5)	
Female	17 (40.5)	5 (45.5)	
Histology			0.74
Grade III	20 (47.6)	4 (36.4)	
Grade IV	22 (52.4)	7 (63.6)	
P53 Overexpression			1.0
Present	22 (52.4)	6 (54.5)	
Absent	20 (47.6)	5 (45.5)	
MIB Labeling			0.59
Very High (>36)	15 (35.7)	2 (18.2)	
Intermediate (18–36)	14 (33.3)	5 (45.5)	
Low (<18)	13 (31.0)	4 (36.4)	
Extent of resection			0.02
<90% resection	24 (60)	2 (18.2)	
≥90% resection	16 (40)	9 (81.8)	