AKT Activation in Human Glioblastomas Enhances Proliferation via TSC2 and S6 Kinase Signaling

Markus J. Riemenschneider, Rebecca A. Betensky, Saskia M. Pasedag, and David N. Louis

Abstract

Aberrant AKT (protein kinase B) signaling is common in many cancers, including glioblastoma. Current models suggest that AKT acts directly, or indirectly via the TSC complex, to activate the mammalian target of rapamycin (mTOR) as the main downstream mediator of AKT signaling. mTOR activation results in subsequent activation of S6K and STAT3, as well as suppression (i.e., phosphorylation) of 4E-BP1, leading to cell cycle progression and inhibition of apoptosis. Most studies of this pathway have used in vitro systems or tumor lysate–based approaches. We aimed to delineate these pathways in a primarily in situ manner using immunohistochemistry in a panel of 29 glioblastomas, emphasizing the histologic distribution of molecular changes. Within individual tumors, increased expression levels of p-TSC2, p-mTOR, p-4E-BP1, p-S6K, p-S6, and p-STAT3 were found in regions defined by elevated AKT activation. However, only TSC2, S6K, and S6 activation levels correlated significantly with AKT activation and clustered together in multidimensional scaling analyses. Ki-67 proliferation indices were significantly elevated in p-AKT-overexpressing regions, whereas expression of the apoptosis marker cleaved caspase 3 was generally low and not significantly different between the regions. These findings provide the first in vivo evidence for a close correlation between AKT and TSC2 phosphorylation levels in glioblastoma. Moreover, they suggest that downstream p-AKT effects are primarily mediated by S6 kinase signaling, thus enhancing proliferation rather than inhibiting apoptosis. (Cancer Res 2006; 66(11): 5618-23)

Introduction

Glioblastoma, the most common and most malignant primary brain tumor (1), often has amplification, rearrangement, and/or overexpression of growth factor receptor genes such as the epidermal growth factor receptor (EGFR) and mutations in the phosphatase and tensin homologue deleted on chromosome 10 (PTEN) gene. Such changes lead, in nearly all glioblastomas, to AKT activation through the phosphatidylinositol 3-kinase (PI3K) pathway (2).

AKT (protein kinase B) is a 56 kDa serine/threonine protein kinase that has emerged as a central player involved in the regulation of cell proliferation, growth, differentiation, migration, and survival in many human cancers. The importance of AKT activation in glioblastomas has been shown in different model systems (3, 4).

In conveying the downstream signaling effects of AKT, the serine/threonine protein kinase, mammalian target of rapamycin (mTOR), has been proposed to play a central role (5). mTOR can be directly activated by p-AKT on its phosphorylation site at Ser2448 (6, 7) or indirectly activated via the tuberous sclerosis complex (TSC), which is formed by hamartin (TSC1) and tuberin (TSC2; refs. 8, 9). As shown in TSC-associated brain lesions, activated AKT can associate with and activate TSC by phosphorylating TSC2 (10), thereby leading to indirect activation of mTOR. The small GTPase Rheb has been identified as a key molecule in mediating the effects of TSC2 on mTOR (11). However, the relevance of this mechanism in glioblastoma remains to be determined.

Two main signaling pathways have been described downstream of mTOR. Activation of ribosomal S6K can enhance translation of mRNAs that bear a 5′-terminal oligopyrimidine tract. The effect of S6K on mRNA translation is indirect via intermediates that are direct downstream effectors of S6K, the most extensively studied of which is ribosomal protein S6 (12). The second established signaling pathway downstream of mTOR is phosphorylation and inactivation of the 4E-BP1 suppressor protein. Phosphorylation of 4E-BP1 leads to its dissociation from the RNA cap-binding protein eIF4E, which facilitates formation of the eIF4F complex. This complex seems to initiate the translation of mRNAs with extensive secondary structure in the 5′-untranslated region (13, 14). In addition to these two primary signaling pathways, mTOR is capable of associating with STAT3 and facilitating STAT3 activation by specific mTOR-dependent phosphorylation of STAT3 at Ser727 (15). Of note, inhibition of mTOR by rapamycin inhibits STAT activation and glial differentiation (16, 17).

Recent studies, however, have questioned the central role of mTOR as the main downstream mediator of AKT signaling, suggesting that the TSC complex might mediate S6K activation independent of mTOR or that S6K (but not AKT) might directly phosphorylate mTOR; this would place mTOR downstream of S6K in the pathway (18, 19). Furthermore, the close relation between AKT and mTOR has been questioned because mTOR is known to integrate a plethora of signaling pathways in response to genetic and metabolic changes (5). As mTOR can be blocked by rapamycin or its ester homologue, CCI-779, and therefore offers a promising treatment option in glioblastoma (20), a better understanding of its role in the AKT pathway is essential.

Most studies of the signaling relationships downstream of AKT have analyzed cell lysates in in vitro systems. We therefore directly investigated AKT activation and the phosphorylation of its signaling intermediates in situ in human glioblastoma samples and correlated the results with proliferation and apoptosis. Because...
many molecules are only focally overexpressed in glioblastomas, we used an in situ approach to investigate topographical relationships between specific phosphorylated molecules in these pathways. We previously showed that this approach is highly suited to dissect the interrelationships of molecules within a given pathway (21) and therefore sought to apply the technique to study the critical issue of AKT signaling in glioblastomas.

Materials and Methods

Materials. Twenty-nine formalin-fixed paraffin-embedded glioblastomas, WHO grade 4 (1), were randomly selected from resection specimens in the Pathology Service, Massachusetts General Hospital, Boston, MA, after appropriate human studies permission was obtained. Histologic evaluation ensured that tumor fragments taken for molecular analysis contained a sufficient proportion (>80%) of tumor cells.

Immunohistochemistry. Immunohistochemistry was done on 5 μm serial sections mounted on poly-€-lysine-coated slides and baked at 60°C overnight. After decervation, slides were treated with 0.5% hydrogen peroxide in methanol for 10 minutes at room temperature. Sections were rehydrated and washed with PBS, followed by antigen retrieval with 10 mmol/L sodium citrate buffer (pH 6) in a microwave oven for 10 to 30 minutes. After blocking with 10% normal goat or horse serum in PBS/1% bovine serum albumin for 30 minutes at room temperature, the sections were incubated overnight at 4°C with the specific primary antibodies. The 10 primary antibodies as well as respective dilutions and positive control tissues are listed in Table 1. On the following day, biotinylated goat anti-rabbit IgG or biotinylated horse anti-mouse IgG at dilutions from 1:250 to 1:1,000 (Vector, Burlingame, CA) was applied for 30 minutes at room temperature, followed by detection of immunoreactivity with an avidin-biotin system (Vector) using either 3,3′-diaminobenzidine tetrahydrochloride or NovaRED (Vector) as a chromogen. Sections were lightly counterstained with Mayer’s hematoxylin (Richard-Allan Scientific, Kalamazoo, MI). Negative controls without primary antibodies were done for all reactions. In addition, negative controls with the respective IgG isotopes were done using negative control antibodies for mouse IgG, mouse IgGκ, and rabbit IgG (LabVision, Fremont, CA). Controls were run under the same conditions and the same IgG concentrations as were used for the respective primary antibodies.

Scoring and interpretation of immunohistochemistry. Given that there was considerable intratumoral heterogeneity for protein activation and expression, in situ correlations between specific molecules were evaluated by recording topographical distributions of expressed proteins within individual tumors. In this regard, blocks had been selected from the central parts of the tumors in order to compare areas of solid tumor tissue. For each case, regions were defined by maximum and minimum expression of AKT (AKTmax and AKTmin). Intact antigenicity in these regions was also scrutinized by staining with a control antibody against vimentin. Slides were then scored blindly for phosphorylation of the downstream AKT signaling intermediates, as well as for the proliferation marker Ki-67 and the apoptosis marker cleaved caspase 3 (cCASP3), in the respective regions.

Two different staining scores were employed. Phosphoprotein expression levels were converted into a composite numerical score (22, 23), based on the percentage of positive stained tumor cells multiplied by staining intensity, potentially ranging from 0 to 12. The percentage of labeled cells was scored as follows: 0 (no or minimal reactivity, similar to nonneoplastic brain tissue), 1 (<10%), 2 (10-50%), 3 (50-90%), 4 (>90%). Staining intensity was graded as 0 (negative), 1 (weak), 2 (moderate), or 3 (strong). Positive vascular staining was not included in the score. Ki-67 and cCASP3 labeling indices were assessed as the mean percentage of positive cells, measured by counting defined areas in five representative high-power fields (magnification, ×400), employing an eyepiece grid. Depending on cell density, ~500 to 4,000 cells were counted per tumor.

Statistical analysis. The Wilcoxon signed rank test was used to compare maximum and minimum regions within tumors. Associations in differentials between maximum and minimum regions were assessed using the normalized differences [(max – min) / max] to adjust for the differences in scale due to different maximum levels. Spearman correlation coefficients were used to assess the correlation between any two ordinal scores. The bootstrap with 2,000 repetitions was employed to calculate 95% confidence intervals (CI) for the correlation coefficients. Adjustment for multiple comparisons was accomplished with Bonferroni corrections. To depict the pairwise relationship among the variables, we used a two-dimensional classical multidimensional scaling plot with a distance metric equal to one minus the absolute value of the Spearman correlation (24).

Results

Regional variability of AKT pathway activation could be detected in all 29 cases. For each case, regions with high (AKTmax) and low (AKTmin) p-AKT expression were selected (Fig. 1). We found a median relative difference in p-AKT protein expression scores of 5 with an interquartile range (IQR) of 3 comparing both regions on a tumor-by-tumor basis in all 29 glioblastomas.

We then assessed differences in the other pathway components within the selected regions (Figs. 1 and 2A). The highest median differences were found for p-TSC2 (median, 4; IQR, 6), p-mTOR (median, 4; IQR, 3), p-S6K (median, 4; IQR, 5), and p-S6 (median, 5; IQR, 3). Median differences for p-4E-BP1 (median, 3; IQR, 4) were slightly lower, mainly due to the lower maximum expression levels of this protein. Only 14 cases had p-STAT3 expression differences, whereas 15 cases had no differences detected between the regions,

| Table 1. Primary antibodies used for immunohistochemical analyses |
|-----------------|--------|-----------------|--------|--------------------|--------|
| **Protein** | **Clone** | **Company** | **Catalog no.** | **Raised in** | **Positive control** | **Dilution** |
| p-AKT (Ser473) | 736E11 | Cell Signaling (Danvers, MA) | 9277 | Rabbit | Prostate cancer | 1:50 |
| p-TSC2 (Thr1462) | Polyclonal | Cell Signaling | 3611 | Rabbit | Breast cancer | 1:10 |
| p-mTOR (Ser2448) | Polyclonal | Cell Signaling | 2971 | Rabbit | Breast cancer | 1:75 |
| p-4E-BP1 (Ser65) | Polyclonal | Cell Signaling | 9451 | Rabbit | Breast cancer | 1:50 |
| p-p70 S6 kinase (Thr389) | 1A5 | Cell Signaling | 9206 | Mouse | Breast cancer | 1:200 |
| p-S6 (Ser253, Ser256) | 91B2 | Cell Signaling | 4857 | Rabbit | Breast cancer | 1:200 |
| p-STAT3 (Ser277) | 6E4 | Cell Signaling | 9136 | Mouse | Breast cancer | 1:2,000 |
| Ki-67 | MIB-1 | Dako (Carpinteria, CA) | M7240 | Mouse | Tonsil | 1:100 |
| cCASP3 (Asp175) | Polyclonal | Cell Signaling | 9661 | Rabbit | Tonsil | 1:200 |
| Vimentin | V9 | BioGenex (San Ramon, CA) | M074-UC | Mouse | Tonsil | 1:100 |
explaining the low median expression differences detected for p-STAT3 (median, 0; IQR, 5). Vimentin staining was used as a positive control to show intact antigenicity of both regions (median, 0; IQR, 0).

First, we tested whether phosphorylation of the single pathway components was dependent on AKT activation by performing Wilcoxon signed rank test analyses (Table 2). Significantly increased expression levels of p-TSC2, p-mTOR, p-4E-BP1, p-S6K, p-S6, and p-STAT3 were found in regions defined by elevated AKT activation (Wilcoxon signed rank test, \( P < 0.0001 \) for p-mTOR, p-4E-BP1, p-S6K, and p-S6; \( P = 0.0003 \) for p-TSC2; \( P = 0.0023 \) for p-STAT3).

We then evaluated which molecules were both increased in p-AKT-overexpressing regions and had phosphorylation levels that significantly correlated with AKT activation levels. Interestingly, only the levels of p-TSC2, p-S6K, and p-S6 expression were significantly correlated with p-AKT expression levels using the normalized measure of difference between p-AKTmax and p-AKTmin regions (\( P < 0.0001 \); 95% CI, 0.492–0.878 for p-TSC2; \( P < 0.0001 \); 95% CI, 0.457–0.838 for p-S6K; \( P = 0.0018 \); 95% CI, 0.224–0.803 for p-S6). In contrast, p-mTOR (\( P = 0.1925 \); 95% CI, −0.127–0.545), p-4E-BP1 (\( P = 0.0527 \); 95% CI, −0.054–0.679), and...
p-STAT3 ($P = 0.0182; 95\% CI, 0.114-0.666$) expression levels were not significantly correlated to AKT activation levels (Table 2).

To provide a visual representation of the pattern of proximitiies (i.e., distances) among the molecules in the pathway, we did multidimensional scaling analyses. The signaling molecules were plotted in two dimensions with the distance between two molecules providing a measure of their interrelatedness. In accord with the univariate regression analyses, p-TSC2, p-S6K, and S6 phosphorylation levels are significantly correlated with AKT activation levels (Spearman correlation). Significant $P$ values captured in italics; $P$ values cutoff for significance, $P \leq 0.005$, correcting for the nine multiple comparisons.

Table 2. Results of the statistical analyses on the differential expression of the six phosphoproteins, Ki-67, cCASP3, and vimentin comparing p-AKTmax/min regions

<table>
<thead>
<tr>
<th>Protein</th>
<th>Spearman coefficients</th>
<th>$P$</th>
<th>Wilcoxon test</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-TSC2</td>
<td>0.75</td>
<td>&lt;0.0001</td>
<td>0.0003</td>
</tr>
<tr>
<td>p-mTOR</td>
<td>0.25</td>
<td>0.193</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>p-4E-BP1</td>
<td>0.36</td>
<td>0.053</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>p-S6K</td>
<td>0.72</td>
<td>&lt;0.0001</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>p-S6</td>
<td>0.55</td>
<td>0.002</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>p-STAT3</td>
<td>0.44</td>
<td>0.018</td>
<td>0.002</td>
</tr>
<tr>
<td>Vimentin</td>
<td>−0.01</td>
<td>0.962</td>
<td>0.172</td>
</tr>
<tr>
<td>Ki-67</td>
<td>n.d.</td>
<td>n.d.</td>
<td>&lt;0.0001</td>
</tr>
<tr>
<td>cCASP3</td>
<td>n.d.</td>
<td>n.d.</td>
<td>0.896</td>
</tr>
</tbody>
</table>

NOTE: Note that expression of p-TSC2, p-mTOR, p-4E-BP1, p-S6K, p-S6, p-STAT3, and Ki-67 is significantly dependent on p-AKT expression (Wilcoxon signed rank test), whereas only TSC2, S6K, and S6 phosphorylation levels are significantly correlated with AKT activation levels (Spearman correlation).

Discussion

Aberrant AKT signaling is common in many cancers, and nearly all glioblastomas show AKT activation through the PI3K pathway (2). Multiple downstream mediators of AKT activation have been suggested by in vitro data (5, 25). However, knowledge about their interrelationship is still preliminary and sometimes controversial.

We correlated the expression of individual molecules across topographical regions directly in human glioblastoma samples. We previously showed that this approach allows direct observational validation of functional relationships between individual pathway components (21).

We compared regions of high and low p-AKT expression on a tumor-by-tumor basis in 29 glioblastoma cases. In agreement with the in vitro studies, the proposed downstream mediators in the AKT signaling pathway—TSC2, mTOR, 4E-BP1, S6K (with the antibody measuring both S6K1 and S6K2), S6, and STAT3—are more likely to be phosphorylated in the same regions in which AKT is activated. These findings imply that these molecules are also regulated by AKT in vivo.

We next asked how closely the phosphorylation levels of these molecules are connected to AKT activation by assessing the degree of correlation between the expression levels of p-AKT and its downstream molecules. Strikingly, only the phosphorylation levels of TSC2, S6k, and S6 correlated significantly with AKT activation, whereas mTOR, 4E-BP1, and STAT3 phosphorylation levels did not.

These data show, for the first time, a close association between TSC2 and AKT phosphorylation in glioblastoma. Although it had been previously shown in TSC-associated brain lesions that activated AKT can associate with and inactivate the TSC complex by phosphorylating TSC2 (8–10), leading to the activation of downstream components in the AKT signaling pathway, the relevance of this mechanism to glioblastoma had not been studied.

These findings also shed light on the role of mTOR within the pathway. Much literature favors mTOR, either directly activated by p-AKT on its phosphorylation site at Ser2448 (6, 7) or indirectly activated via TSC, as the main downstream mediator of AKT signaling; as a result, mTOR activates S6 kinase signaling and phosphorylates 4E-BP1 and STAT3, leading to increased proliferation and inhibition of apoptosis (5). However, recent in vitro studies provide evidence that the TSC complex might mediate S6K and consecutive S6 activation independent of mTOR, or that S6K (but not AKT) might directly phosphorylate mTOR; such data would place mTOR downstream of S6K in the pathway (18, 19). In turn, the lack of significant correlation of p-4E-BP1 and p-STAT3 with p-AKT fits well with the current model that places these molecules downstream of p-mTOR.

Based on our in situ observations, the smaller degree of correlation between p-mTOR and p-AKT is not likely due to a further downstream location of p-mTOR in the pathway. mTOR activation levels were generally high, and p-mTOR protein expression in seven cases was even observed in the complete absence of AKT activation in the AKTmin regions. These data therefore support the notion that mTOR is influenced by additional...
signaling pathways in response to genetic and metabolic changes independently of AKT (26–29).

Although AKT activation can relieve TSC repression of Rheb and activate mTOR (Fig. 3), AKT-independent mTOR regulation pathways include p90RSK, which can inhibit TSC2 in response to growth factors (30), as well as LKB1 and REDD1, which can activate TSC2 in response to low ATP levels and hypoxia, respectively (31, 32). In addition, mTOR is found in two structurally and functionally distinct complexes, mTORC1 and mTORC2, further adding to the complexity of this pathway (33). mTORC1 is rapamycin-sensitive and functions upstream of S6K and 4E-BP1. mTORC2, on the other hand, controls the actin cytoskeleton, is rapamycin-insensitive, is under regulation by nutrient and growth factor cues, and is not definitively downstream of the TSC complex and Rheb (34). Because the antibody used in this study cannot distinguish between the two mTOR complexes, it remains possible that the lack of correlation between AKT and mTOR activation in this study could be due to the convergence of multiple signaling pathways on mTOR regulation via two different mTOR complexes.

Using multidimensional scaling analysis, in which the distance between molecules is regarded as a measure of their interrelatedness (24), we observed clustering of p-TSC2, p-S6K, and p-S6 in close proximity to p-AKT, which favors close interrelationships of these molecules in conveying downstream p-AKT signaling. In contrast, p-mTOR, p-4E-BP1, and p-STAT3 mapped more distant from p-AKT in the plot. The lack of an observed cluster between p-mTOR, p-4E-BP1, and p-STAT3 does not argue against functional relations between these molecules; we intentionally biased the selection of regions for differences in AKT activation, and therefore only derive clustering data regarding AKT activation.

Most studies of the signaling relationships downstream of AKT have used in vitro systems and knowledge of the in vivo pathway is primarily based on tumor lysate–based approaches. Only one previous study used a histologic approach to study the pathway in glioblastoma (35): this analysis focused on delineating the entire PI3K signaling pathway, and overlapping data only exist for p-mTOR and p-S6. In line with our results, this study found a close correlation between p-AKT and p-S6 expression, but also reported a correlation between p-AKT and p-mTOR. Differences in techniques could account for the divergent findings; their study used tissue microarrays, which may not account for the heterogeneous nature of glioblastoma. Given the regional differences we detected for protein expression within tumors, the use of tissue microarrays could pose a risk of sampling error because only small pieces of tissue are present in the arrays.

Because AKT activation is thought to enhance proliferation and inhibit apoptosis of tumor cells (36, 37), we also compared the expression levels of the proliferation antigen Ki-67 (38) and the apoptosis marker cCASP3 (39, 40) between the regions. Ki-67 labeling indices were elevated in p-AKT-overexpressing regions. cCASP3 expression, in contrast, was generally low and not different between regions. These findings suggest that the oncogenic effect of AKT activation is most likely a result of enhancing tumor cell proliferation in glioblastoma.

In summary, the topographical relationships between AKT signaling components detected in this study provide the first in vivo evidence for a close correlation between AKT and TSC2 phosphorylation levels in glioblastoma. Moreover, AKT activation seems more closely related to S6K and S6 than to mTOR activation, suggesting that the downstream effects of AKT activation are primarily conveyed by TSC2 and S6 kinase signaling and that they enhance proliferation rather than inhibit apoptosis. These findings stress the potential importance of S6 kinase signaling downstream of AKT and raise the possibility that it may be a more relevant therapeutic target than mTOR in glioblastomas with AKT activation. Nonetheless, the results leave open the possibility that mTOR, which can be blocked by rapamycin or its ester homologue, CCI-779, could be a therapeutic target in glioblastoma (34). At the same time, these results predict that activation of the AKT signaling pathway in glioblastoma may not be an appropriate selection criterion for including patients in mTOR inhibitor trials.

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References

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