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Analysis of the activation status of Akt, NFκB, and Stat3 in human diffuse gliomas

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Loss of phosphatase and tensin homolog (PTEN) and amplification of the epidermal growth factor receptor (EGFR) gene contribute to the progression of gliomas. As downstream targets of the PTEN and EGFR signaling pathways, Akt, NFκB, and signal transducer and activator of transcription-3 (Stat3) have been shown to play important roles in the control of cell proliferation, apoptosis, and oncogenesis. We examined the activation status of Akt, NFκB, and Stat3 in 259 diffuse gliomas using tissue microarrays and immunohistochemistry, and evaluated their association with glioma grade. We observed significant positive correlations between the activation status of Akt and NFκB and glioma grade. In contrast, only focal immunoreactivity for phospho-Stat3 was observed in <9% of high-grade gliomas. In addition, we observed a significant correlation between the activation of Akt and NFκB. Functional correlation between Akt activation and the activation of NFκB was confirmed in U251MG GBM cells in which inhibition of Akt activation either by stable expression of PTEN or by the PI3-kinase inhibitors, wortmannin and LY294002, led to a concomitant decrease in NFκB-binding activity. Thus, our results demonstrate that constitutive activation of Akt and NFκB, but not Stat3, contributes significantly to the progression of diffuse gliomas, and activation of Akt may lead to NFκB activation in high-grade gliomas.
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Diffuse gliomas constitute the most common type of intracranial malignant neoplasm and account for more than 60% of all primary brain tumors.[1] The World Health Organization (WHO) classification of tumors of the nervous system separates diffuse gliomas into seven principal categories: diffuse astrocytoma (LGA, WHO Grade II), oligodendroglioma (O, WHO Grade II), oligoastrocytoma (MOA, WHO Grade II), anaplastic astrocytoma (AA, WHO Grade III), anaplastic oligodendroglioma (AO, WHO Grade III), anaplastic oligoastrocytoma (AMOA, WHO Grade III), and glioblastoma (GBM, WHO Grade IV).[1] Significant differences exist in patient survival between the different histologic grades of diffuse glioma. The median survival for LGA is greater than 5 years, compared to 2–5 years for AA, and less than 1 year for GBM.[2–4]

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Many alterations in the genes that regulate normal homeostasis of cell proliferation, differentiation, and apoptosis contribute to the formation and progression of diffuse gliomas.[1] Among these genetic lesions, amplification of the epidermal growth factor receptor (EGFR) gene and mutation or deletion of the phosphatase and tensin homolog (PTEN) gene occur in 40–50% of high-grade gliomas, particularly in primary GBM.[5–8] EGFR is a transmembrane receptor with intrinsic tyrosine kinase activity that is normally regulated by ligands binding to its extracellular domain. In GBM, amplification of EGFR and in-frame deletion of exons 2–7 of the EGFR gene, which results in a constitutively active form of EGFR, activate several signaling pathways, including the MAP kinase, PI3 kinase/Akt, and Jak/Stat pathways.[9,10]

PTEN is a phospholipid phosphatase that dephosphorylates phosphatidylinositol 3,4,5-triphosphate.[11–13] As such, PTEN inhibits PI3-kinase-dependent activation of Akt. Conversely, mutation or loss of PTEN leads to constitutively activated Akt. In turn, activated Akt phosphorylates and inactivates Bad, caspase 9, and Forkhead transcription

factors, which contribute not only to the suppression of apoptosis but also to the promotion of cell survival.[14–16] PTEN is expressed at a significantly lower level in GBMs than in gliomas of lower grades. Independent of age and tumor grade, patients with gliomas expressing low levels of PTEN have a significantly poorer prognosis than do those with higher levels.[17] PTEN has also been shown to regulate tumor-induced angiogenesis in GBM cell xenografts.[18] Akt activity is elevated in GBM cells expressing mutant forms of PTEN.[13] In animal studies, activation of Akt has been shown to be essential for the formation of GBMs from genetically modified neural progenitors and normal human astrocytes, suggesting that activation of Akt may play an important role in glioma formation and progression.[19,20]

Among many of its biological effects, activated Akt has been shown to phosphorylate and activate IκB kinase (IKK), which, in turn, phosphorylates IκB and leads to its degradation and to the nuclear translocation of NFκB.[21–23] NFκB is a heterodimeric transcription factor consisting predominantly of p65/RelA and p50 subunits. NFκB is normally sequestered in the cytoplasm through its interaction with proteins belonging to the IκB family, which inhibits the translocation of NFκB into the nucleus. Stimulation by cytokines or growth factors leads to rapid phosphorylation of IκB by IKK. Phosphorylated IκB is rapidly ubiquinated and degraded through the 26S proteasome pathway.[24] The degradation of IκB liberates NFκB and allows it to translocate into the nucleus where it regulates the transcription of target genes.[24] It has also been shown that the p65 NFκB subunit is phosphorylated by IKK and by the PI3-kinase/Akt pathway, which leads to the activation of NFκB.[25–28] In human glioma cell lines, NFκB is constitutively activated and confers resistance to TNF-α-induced apoptosis.[29,30] Conversely, inhibition of NFκB activation prevents cell cycle progression and inhibits the growth of U251MG glioma cells treated with TNF-α.[29] NFκB has also been reported to be constitutively activated in other human cancers, including breast carcinoma, pancreatic adenocarcinoma, acute lymphoblastic leukemia, and high-grade gliomas, especially GBM.[30–34] It has been speculated that constitutive activation of NFκB in GBM may be associated with tumor resistance to TNF-α immunotherapy.[34] These findings suggest that NFκB activation could play an important role in the tumorigenesis of diffuse gliomas and in promoting the growth of high-grade gliomas. Previous studies on the activation of Akt and NFκB in diffuse gliomas, however, have been limited primarily to cultured cell lines or to relatively small numbers of tumors, and no large-scale studies have examined the activation of Akt and NFκB in human glioma tissues. It is also not clear whether activation of Akt correlates with the activation of NFκB in diffuse gliomas.

The signal transducer and activator of transcription (Stat) proteins constitute a family of latent transcription factors activated by a number of cytokines and growth factors, including epidermal growth factor (EGF) and interleukin-6. Stat proteins, particularly Stat3 and Stat5, have been reported to be constitutively activated in several cancers and cancer-derived cell lines.[35] Despite the established connection between EGFR and Stat activation, the role of the Jak/Stat pathway in glioma progression is still not clear.

In this study, we used high-throughput tissue microarrays and immunohistochemistry to examine the activation status of Akt, NFκB, and Stat3 in 259 human diffuse gliomas, and correlated the results with glioma grade. We also examined the functional correlation between Akt activation and the activation of NFκB in diffuse gliomas. Furthermore, we examined the effect of inhibition of Akt on NFκB-binding activities in U251MG GBM cells either by stable expression of PTEN or by the PI3-kinase inhibitors wortmannin and LY294002. This study provides the first dissection of two important signal transduction pathways in diffuse gliomas *in vivo* and offers new insights potentially useful for the design of mechanism-based therapies for GBM.

Materials and methods

Stable Expression of PTEN in U251MG and LN229 GBM Cell Lines

PT67 retrovirus producer cells were grown in Dulbecco's modified Eagle's medium/F12 containing 10% fetal calf serum, 1000 U/ml penicillin–streptomycin and 2 mM glutamine, and transfected with pLNCX retrovirus harboring full-length PTEN cDNA by calcium phosphate precipitation. The LN229 and U251MG GBM cells were infected with 48-h supernatants from the transfected PT67 cells. The stable clones were selected with 400 μg/ml G418 and screened for PTEN expression by immunoblotting.

Selection of Diffuse Glioma Samples and Construction of Glioma Tissue Microarray

Glioma samples were obtained from patients who had undergone surgery at our institution from 1986 through 2001. The formalin-fixed, paraffin-embedded archival tissue blocks were retrieved, and the matching hematoxylin and eosin (H&E)-stained slides were reviewed and screened for representative tumor regions by a neuropathologist. Glioma samples were then grouped according to the diagnostic criteria of the WHO 2000 classification system. The composition of tumors included in the tissue microarray is listed in Table 1. A tissue microarray was constructed from the selected gliomas with a tissue microarrayer (Beecher

Table 1 Composition of tumor types in glioma tissue microarray

Diffuse gliomas	*Number of cases (tissue cores)*
Glioblastoma (GBM, WHO Grade IV)	70 (140)
Anaplastic astrocytoma (AA, WHO Grade III)	49 (98)
Diffuse astrocytoma (LGA, WHO Grade II)	9 (18)
Anaplastic oligodendroglioma (AO, WHO Grade III)	41 (82)
Oligodendroglioma (O, WHO Grade II)	41 (82)
Anaplastic mixed oligoastrocytoma (AMOA, WHO Grade III)	15 (30)
Oligoastrocytoma (MOA, WHO Grade II)	24 (48)
Gliosarcoma (GS, WHO Grade IV)	10 (20)
Normal brain (NB)	10 (20)
Control cell lines (CL)	16 (16)

Instruments, Sun Prairie, WI, USA) as described previously.[36] The tissue microarray included 259 primary gliomas representing all histologic types and grades of diffuse glioma codified in the WHO classification system. Each tumor was sampled in duplicate from representative areas of either one or two donor blocks using a 0.6-mm punch, yielding a composite (array) block comprising a total of 555 tissue cores. In addition, 10 normal brain samples from surgical resections and 16 cultured cell lines (10 GBM, two breast cancer, two squamous cell carcinoma, one colon cancer, and one fetal kidney) were also included as negative and positive controls. Normal brain tissue samples were obtained from surgical resections. Each normal brain was sampled with two 0.6-mm tissue cores to include one core from normal white matter and one from gray matter. We specifically included LN229 and U251HF GBM cells that stably express PTEN as negative controls for pAkt immunohistochemical staining. U87MG and U373 GBM cells treated with TNF-α were included as a positive control for NFκB activation.

Immunohistochemistry

Anti-phospho-Akt (Ser^{473}), a polyclonal antibody that detects Akt only when phosphorylated at Ser^{473}, anti-phospho-NFκB p65 (Ser^{536}) antibody, which is a polyclonal antibody that recognizes p65 only when phosphorylated at Ser^{536}, and anti-phospho-Stat3, which is a monoclonal antibody that detects Stat3 only when it is phosphorylated at Tyr^{705}, were purchased from Cell Signaling Technology (Beverly, MA, USA). Anti-NFκB p65 antibody, a polyclonal antibody directed against the N-terminal domain of the human NFκB p65 subunit, was purchased from Santa Cruz Biotechnology (SC-109; Santa Cruz, CA, USA).

A standard indirect immunoperoxidase procedure (Elite-ABC kit; Vector Laboratories, Burlingame, CA, USA) was used for all staining. In brief, antigen retrieval was performed by treating the unstained slides in a steamer for 25 min. Antibodies against phospho-Akt (pAkt, 1:50), NFκB p65 subunit (1:250), phospho-NFκB p65 (Ser^{536}) (pNFκB p65, 1:50), or phospho-Stat3 (pStat3, 1:100) were overlaid on glioma array tissue sections and incubated overnight at 4°C. Secondary antibody incubation was performed at room temperature for 60 min. Mayer's hematoxylin nuclear staining was used as a counterstain. The results of immunohistochemical staining for pAkt, pStat3, NFκB, and pNFκB p65 subunit were evaluated independently by two pathologists and placed into one of two categories: positive or negative. The positive category represented gliomas exhibiting a strong positive reaction in at least 5% of the tumor cells in one tissue core, whereas the negative category represented tissue samples with very weak or absent staining. Cases with weak staining were always in less than 5% of the tumor cells.

Western Blot

Western blot analysis was used to examine the activation status of Akt and NFκB in GBM cells and frozen tissue samples from normal brains and GBMs. Briefly, U251MG cells were treated with control medium or with wortmannin or LY294002 for 3 h before harvest for cell extract preparation. All tissue samples used for Western blots were selected by neuropathologists from surgical resections based on frozen sections prepared from frozen tissue blocks. Equal amounts of proteins were resolved by 10% SDS-PAGE and electroblotted onto Hybond ECL nitrocellulose membranes (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The filters were blocked in 5% skim milk in $1\times$TBS and then probed with anti-Akt, anti-pAkt (Ser^{473}), or anti-pNFκB p65 (Ser^{536}) antibodies and detected with an enhanced chemiluminescence kit (ECL, Amersham).

NFκB DNA-Binding Assay

Electrophoretic mobility shift assays (EMSA) were performed as described previously.[37] Briefly, nuclear extracts were prepared from U251MG cells treated with control medium or with the PI3 kinase inhibitors wortmannin and LY294002. Nuclear proteins were incubated with radiolabeled NFκB-binding oligonucleotides (2×10^4 c.p.m.) as probe and nonspecific competitor poly(dI:dG). After incubation for 30 min, protein–DNA complexes were resolved on 5% polyacrylamide gels and visualized by autoradiography.

Statistical Analysis

The results of immunohistochemical staining were analyzed using the χ^2-test or Fisher's exact test,

performed using the SigmaStat (version 2.0, SPSS, Chicago, IL, USA). $P<0.05$ is considered significant.

Results

Immunohistochemical staining for pAkt, NFκB, pNFκB p65, and pStat3 in glioma tissue microarrays was technically successful in 98, 95, 98, and 98% of the tumors, respectively. Representative micrographs of immunostaining for pAkt, NFκB p65, pNFκB p65, and pStat3 in normal brain cortex and gliomas are shown in Figure 1. In normal brain tissues, pAkt was positive only in neurons, while NFκB and pNFκB p65 were positive only in endothelial cells. Astrocytes and oligodendrocytes were negative for pAkt, NFκB, and pNFκB p65 in all 10 normal brain samples examined (Figure 1a, c, and e). No immunoreactivity was observed for pStat3 in all 10 normal brains (Figure 1g). In gliomas, however, tumor cells showed diffuse cytoplasmic and scattered nuclear staining for pAkt, NFκB p65 and pNFκB p65. Immunostaining using anti-pStat3 antibody showed only focal nuclear staining in gliomas (Figure 1b, d, f, and h).

Activation of Akt in Gliomas

To determine whether correlation exists between activation of Akt and the histologic grade of glioma, the percentages of gliomas positive for pAkt in different histologic types and grades were determined and compared. As shown in Figure 2a, pAkt was positive in 84% (59/70), 44% (20/46), and 22% (2/9) of GBMs, AAs, and LGAs, respectively. Paired comparisons between GBMs and AAs, GBMs and LGAs, and AAs and LGAs showed statistically significant differences in the frequencies of Akt activation ($P<0.001$). Similarly, comparisons between the anaplastic forms and the low-grade forms of the oligodendrocytic and the oligoastrocytic series also showed statistically significant differences in activation of Akt ($P<0.05$) (Figure 2a). pAkt was overexpressed in 51% (21/41) of AOs and 53% (8/15) of AMOAs, as compared to 18% (7/40) of Os and 8% (2/24) of MOAs. The frequencies of Akt activation in anaplastic groups of oligodendrocytic or oligoastrocytic tumors were significantly higher than those in Os or MOAs ($P<0.05$). There were, however, no significant differences in Akt activation among AAs, AOs, and AMOAs, or among LGAs, Os, and MOAs. It is interesting that pAkt was also strongly positive in all gliosarcomas examined (10/10) (Figure 2a). Activated Akt was not observed in astrocytes or oligodendrocytes of normal brain cortex or cerebellum. To further confirm our immunohistochemical staining data, we performed Western blot analysis using frozen tissue samples from three normal brains and 14 GBMs. Of 14 GBMs, 10 (71%) showed high levels of pAkt (Figure 3). The other four GBMs also showed low, but detectable, levels of activated Akt. No pAkt was, however, detected in the normal brain tissues (Figure 3). These results show that activation of Akt significantly correlates with histologic grade in diffuse gliomas and the frequency of activated Akt increases with glioma progression.

Overexpression and Activation of NFκB in Gliomas

Immunostaining using anti-p65 antibody, which detects both the active and inactive NFκB p65 subunit, showed diffuse cytoplasmic staining with scattered nuclear staining in gliomas but was negative in the astrocytes and oligodendrocytes of normal brains. Using this antibody, NFκB p65 was shown to be overexpressed in 81% (56/69) of GBMs, 65% (32/49) of AAs, and 14% (1/7) of LGAs. All 10 gliosarcomas also showed strong staining for NFκB p65 (Figure 2b). The difference in the frequency of NFκB p65 overexpression between GBMs and AAs was not statistically significant. High-grade astrocytomas showed a significantly higher frequency of NFκB overexpression when compared to LGAs ($P<0.05$). NFκB p65 was overexpressed in 77% (30/39) of AOs and 67% (10/15) of AMOAs. Both of these expression rates were significantly higher than those of Os (38%, 14/37) and MOAs (38%, 8/21) (Figure 2b). Thus, these data show a strong positive correlation between overexpression of NFκB p65 and histologic grade in diffuse gliomas.

To determine whether elevated NFκB p65 in diffuse gliomas represents the active form of NFκB, immunohistochemistry was performed using a polyclonal antibody directed against human pNFκB p65 (Figure 2b). This antibody detects activated NFκB p65 only when it is phosphorylated at Ser^{536}.[25] pNFκB p65 was detected in 91.3% of GBMs, which was significantly higher than in anaplastic gliomas (AAs, 73.3%; AO, 76.9%; and AMOA, 66.7%), LGAs (37.5%), Os (50%), and MOAs (50%) ($P<0.05$). The positive rates for pNFκB p65 in AAs and AOs were significantly higher than in LGAs, Os, and MOAs ($P<0.05$). There was, however, no significant difference in pNFκB p65 among AAs, AOs and AMOAs, or among LGAs, Os and MOAs. Nine of 10 gliosarcomas examined were also positive for pNFκB p65 (Figure 2b). To further confirm the activation of NFκB in diffuse gliomas, Western blots were performed using the same antibody against pNFκB p65 on frozen tissue samples from normal brains and GBMs. The Western blots showed that pNFκB p65 was detectable in 93% of the GBMs examined (13/14), but not in the three normal brain samples (Figure 3). Therefore, our data consistently show that NFκB is not only overexpressed but also activated in diffuse gliomas. Moreover, the degree of overexpression and activation of NFκB correlate with glioma grade, with high-grade gliomas showing higher levels of activated NFκB compared to low-grade gliomas.

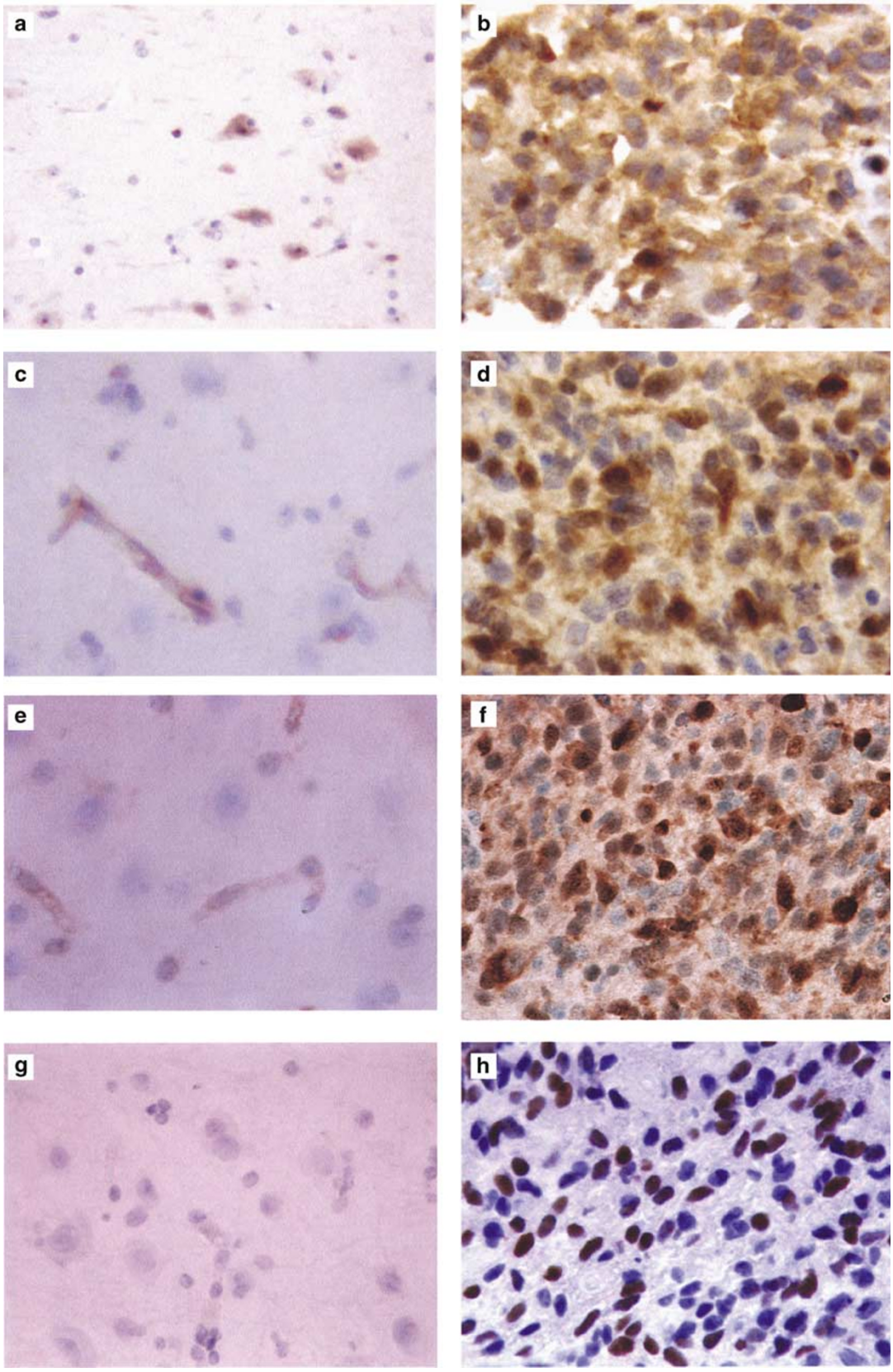

Figure 1 Expression of pAkt, NFκB p65, pNFκB p65, and pStat3 in normal brain cortex and gliomas. Representative micrographs of immunostaining are shown (original magnification, × 200). Expression of pAkt in normal brain cortex (**a**) and GBM (**b**); expression of NFκB p65 in normal brain cortex (**c**) and GBM (**d**); expression of pNFκB p65 in normal brain cortex (**e**) and GBM (**f**); expression of pStat3 in normal brain cortex (**g**) and AO (**h**).

Expression of pStat3 in Gliomas

Considering the well-established connection between the EGFR and Stat signaling pathways, we chose to examine the activation of Stat3 in gliomas using a monoclonal antibody that detects Stat3 only when phosphorylated at Tyr^{705}. pStat3 was detected in 9% (6/70), 8% (4/46), and 12.5% (1/8) of GBMs, AAs, and LGAs, respectively. Among the 254 gliomas for which data were available, only 23 cases (9%) showed focally positive nuclear staining for pStat3. There was no correlation between histologic grade and pStat3 expression (Figure 2c). In contrast to the diffuse immunoreactivity seen for pAkt, NFκB p65, and pNFκB p65, the immunoreactivity for pStat3 was focal in all 23 positive gliomas. In 17 of the 23 gliomas that showed positivity for pStat3, focally positive nuclear staining was present in only one of the two tissue cores from the same tumor.

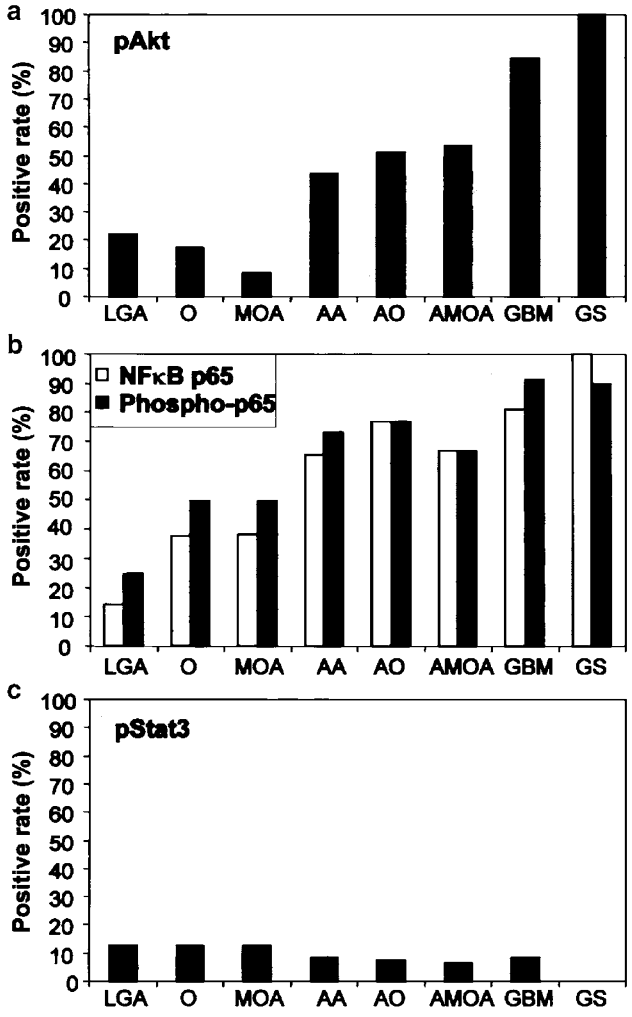

Figure 2 Activation of Akt, NFκB, and Stat3 in human gliomas. The percentage of positive tumors *vs* the histologic types and grades was plotted. (**a**) Activation of Akt correlates with histologic grade in diffuse gliomas. (**b**) Overexpression and activation of NFκB correlates with histologic grade in diffuse gliomas. (**c**) Focal expression of pStat3 in diffuse gliomas.

Activation of Akt in Diffuse Gliomas Contributes to the Activation of NFκB

When the activation status of Akt and NFκB was compared, a significant positive correlation was observed ($P<0.001$, Figure 4). Among the 122 gliomas that were positive for pAkt, 108 (89%) also showed positive staining for activated NFκB. In contrast, only 53% of the gliomas that were negative for pAkt showed positive staining for pNFκB (Figure 4). Consistent with this was the fact that 10 of 10 GBMs (100%), which had high levels of pAkt, also showed increased levels of pNFκB by Western blot (Figure 3). It is noteworthy that nine of 10 gliosarcomas that had elevated pAkt also showed positive staining for pNFκB. A similar correlation between the expression of pAkt and NFκB was also obtained using antibody directed against NFκB p65 (data not shown). Therefore, our data demonstrate that activation of Akt may play a role in the activation of NFκB in high-grade gliomas.

To confirm whether activated Akt in high-grade gliomas contributes to the activation of NFκB, we examined the activation status of Akt and NFκB in two GBM cell lines, U251MG and U251MG/PTEN, which were included in our glioma tissue microarray. U251MG/PTEN cells were derived from U251MG cells by the stable expression of PTEN. When probed with anti-pAkt, anti-NFκB p65, or

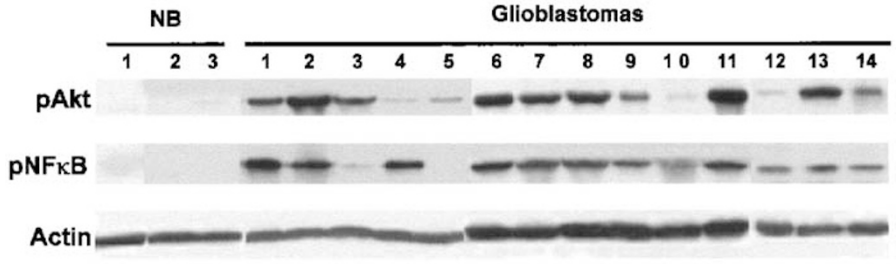

Figure 3 Activation of Akt and NFκB in normal brain and GBM. Tissue samples for normal brain and GBMs were selected by a neuropathologist based on examination of frozen sections prepared from frozen tissue blocks. Tissue extracts from normal brains (NB) and GBMs were Western blotted and probed with anti-pAkt (top panel), anti-pNFκB p65 (middle panel), and antibody against β-actin (bottom panel).

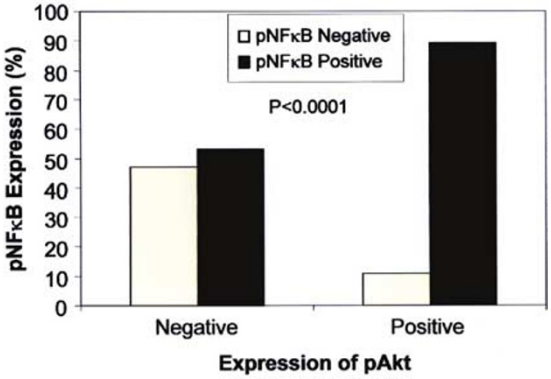

Figure 4 Expression of pAkt correlates with the expression of pNFκB p65 in diffuse gliomas.

anti-pNFκB p65 antibodies, U251MG cells showed an immunostaining pattern similar to that of glioma tissue samples: diffuse cytoplasmic staining with scattered nuclear staining (Figure 5a). The strong immunostaining for activated Akt and pNFκB p65 in U251 MG cells was confirmed by Western blotting of cell extracts using anti-pAkt antibody (Figure 5b) and by EMSA using ^{32}P-labeled NFκB probes (Figure 5c). Expression of PTEN in U251MG/PTEN cells led to a marked decrease in activated Akt as determined by immunohistochemical staining (Figure 5a) or Western blotting (Figure 5b). Inhibition of Akt activation in U251MG/PTEN cells resulted in a concomitant decrease in NFκB DNA binding activity (Figure 5c). To further examine the role of activated Akt in the activation of NFκB, two well-characterized PI3-kinase inhibitors, wortmannin and LY294002, were used to inhibit Akt, and the effect on NFκB-binding activities was examined. Treatment of U251MG cells with wortmannin or LY294002 resulted in a dose-dependent decrease in the levels of pAkt (Figure 6a). These cells also showed a concomitant dose-dependent decrease in NFκB-binding activities (Figure 6b). Similar results were obtained with U87MG and U373 cells (data not shown). These results strongly suggest that activated Akt in GBM cells contributes to the activation of NFκB.

Discussion

Using tissue microarrays and immunohistochemistry, we examined 259 gliomas and found that both Akt and NFκB were consistently activated in diffuse gliomas, but not in the astrocytes or oligodendrocytes of normal brain cortex and cerebellum. We observed a strong positive correlation between the activation status of Akt and NFκB and glioma histologic grade. These findings were confirmed by Western blot using frozen tissue from normal brains and GBMs. In contrast, focal nuclear immunoreactivity for pStat3 was observed only in a small percentage of gliomas examined. These findings indicate that activation of Akt and NFκB, but not Stat3, may play an important role in the formation and progression of human diffuse gliomas. In addition, we also observed a strong correlation between the activation of Akt and the activation of NFκB, which was observed in high-grade glioma tissue samples and in GBM cell lines, suggesting that activation of Akt may lead to the activation of NFκB in gliomas.

The degree of heterogeneity and the expression pattern of the markers under study constitute a potential problem in tissue microarray-based studies, particularly in the case of GBM, a tumor whose molecular heterogeneity has been documented for many markers. The representativity of tissue microarray-based studies in general, however, has been extensively examined, and the data published so far have clearly indicated that the frequencies of molecular alterations identified by high-throughput tissue microarray screening analysis generally correspond well with those identified using full-size tissue sections.[38] For example, good agreement among immunostaining results for p53 in diffuse gliomas has been shown in a comparison study of full-size tissue sections with tissue microarray analysis using single 0.6-mm cores.[39] To increase representativity, we have taken special precaution to select only representative tumor areas and incorporated two cores from each glioma sample into the tissue microarray. The concordant rates for immunostaining between the two tissue cores were 98% for pAkt and 94% for NFκB and pNFκB p65. Among 259 diffuse gliomas examined, only five cases (1.9%; two GBMs, one AA, and two AOs) showed discordant expression of pAkt between the two cores. A slightly higher tissue core discordance rate was observed for NFκB immunostaining (6%, 16/259). Discordant expression was observed only in high-grade gliomas. This may, in part, be due to the degree of heterogeneity in these tumors. For the cases with core discordance, the case was considered positive if one of the cores showed strong positive staining. In addition, we specifically included normal brain tissue cores and cytospin cores of genetically altered cells and cytokine-treated cells in our tissue microarray to serve as positive or negative controls. Such controls help to ensure the reliability of interpretation of the immunohistochemical data.

Akt, a serine/threonine kinase, belongs to a small gene family composed of three highly homologous members: Akt1, Akt2 and Akt3. The pAkt (Ser^{473}) antibody used in this study detected all three forms, but only when they are phosphorylated at equivalent sites. Activation of Akt by the PI3-kinase pathway is inhibited by PTEN. U251MG/PTEN GBM cells, which stably express PTEN, showed negative immunostaining on our tissue microarray (Figure 5). U373 and U87MG, which contain mutations or deletions of PTEN, showed strong cytoplasmic and nuclear staining of pAkt (data not shown).

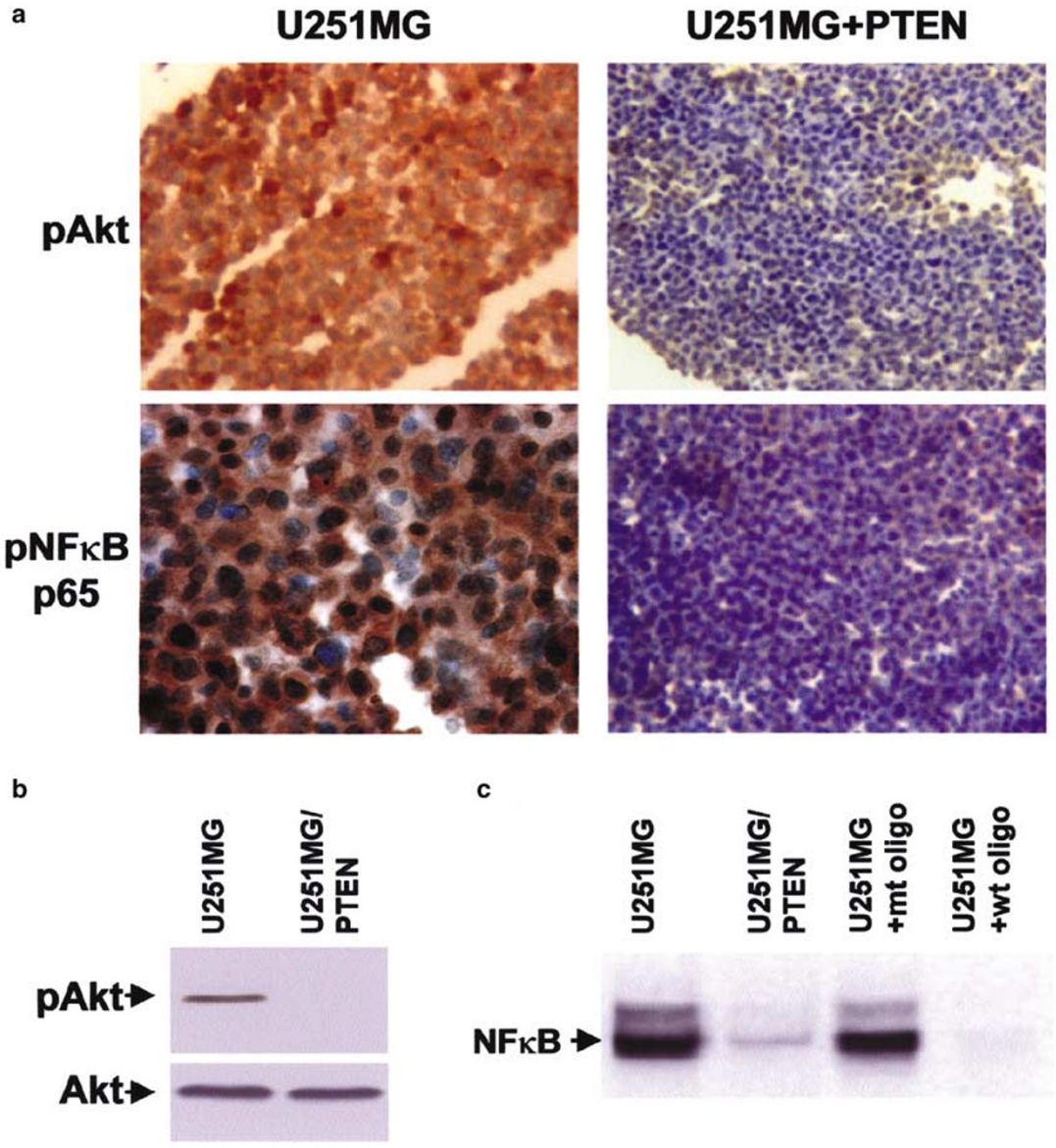

Figure 5 Expression of PTEN in U251MG cells inhibits Akt activation and NFκB binding activity. (**a**) Levels of pAkt and pNFκB p65 are reduced in U251MG/PTEN cells. U251MG and U251MG/PTEN cells were stained with anti-pAkt and anti-pNFκB p65 antibodies. (**b**) U251MG/PTEN cells have reduced activated Akt. Cell extracts from U251MG and U251MG/PTEN cells were Western blotted and probed with anti-pAkt and anti-Akt antibodies. (**c**) U251MG/PTEN cells have reduced NFκB-binding activity. Nuclear extracts from U251MG and U251MG/PTEN cells were used in EMSA, with no competitor or with either wild-type (wt) or mutated (mt) NFκB-binding oligonucleotides (oligo). Position of the NFκB:DNA complex is indicated by the arrow.

It is of interest that both cytoplasmic and nuclear staining was observed in glioma tissue samples. Nuclear translocation of Akt has been shown to be an important step in Akt-mediated cell proliferation and antiapoptosis.[40–42] Our immunostaining results revealed statistically significant differences in the frequencies of pAkt expression among different grades of gliomas. In contrast, no pAkt immunostaining was detected in astrocytes or oligodendrocytes of 10 normal brain controls. Consistent with these findings is the fact that increased levels of pAkt were detected by Western blot in all 14 GBMs, but not in normal brains. Therefore, our data strongly suggest that activation of Akt correlates with glioma grade and support a role for Akt in glioma formation and progression.

Immunostaining for the NFκB p65 subunit with a polyclonal antibody that recognizes both active and inactive p65 showed diffuse cytoplasmic staining with scattered nuclear staining in gliomas. To further confirm the activation status of NFκB in diffuse gliomas, we performed immunohistochemical staining and Western blot analysis using a polyclonal anti-pNFκB p65 antibody specific for activated NFκB p65. Phosphorylation of the p65 NFκB subunit by IKK occurs during the phospho-

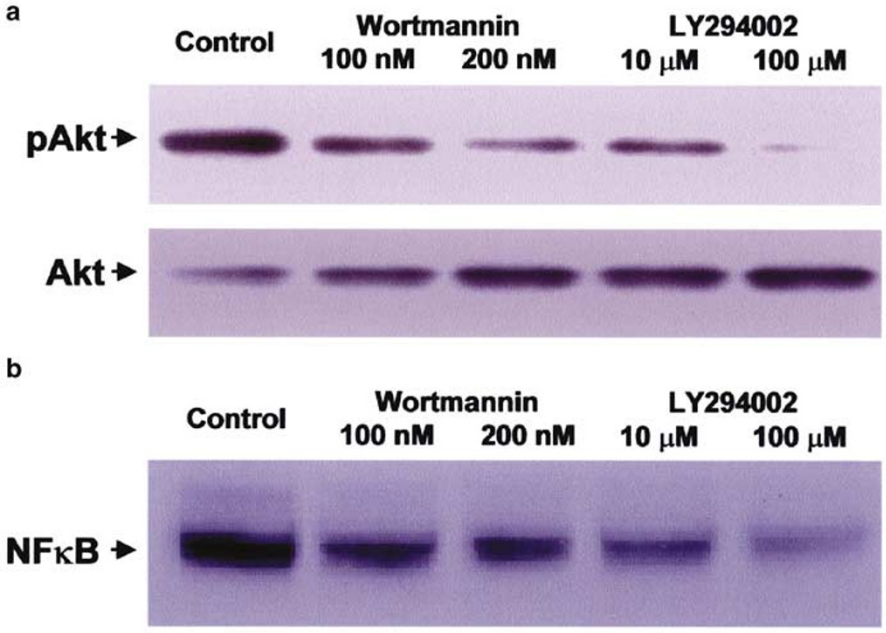

Figure 6 Wortmannin and LY294002 inhibit Akt activation and NFκB-binding activity in U251MG cells. (**a**) Cell extracts from control U251MG cells or cells treated for 3 h with wortmannin or LY294002 were probed with anti-pAkt or anti-Akt antibodies. (**b**) Nuclear extracts from cells treated as in (**a**) were incubated with ^{32}P-labeled NFκB-binding oligonucleotides in EMSA.

rylation and degradation of IκBs and leads to the activation of NFκB.[25] Using this antibody, we observed a similar immunostaining pattern and a significantly higher frequency of NFκB activation in GBMs than in AAs and LGAs. No immunostaining was observed in astrocytes or oligodendrocytes in the 10 normal brain tissue samples included in our tissue microarray using either an anti-NFκB p65 or an anti-pNFκB p65 antibody. Therefore, our immunohistochemical staining data show that NFκB p65 is not only overexpressed but is also activated in diffuse gliomas compared to astrocytes or oligodendrocytes. This conclusion is further supported by increased expression of pNFκB p65 in GBMs (93%) by Western blot and increased NFκB-binding activity in GBM cell lines. While it is unclear why most activated NFκB remains in the cytoplasm, Miyamoto *et al*[43] have reported that only 10–20% of activated NFκB is located in the nucleus when NFκB is activated by long-term stimulation in differentiating B cells. The frequency of NFκB activation in anaplastic forms of gliomas was significantly lower than in GBMs. AAs and AOs (WHO grade III) had significantly higher frequencies of NFκB activation than grade II gliomas (LGA, Os, and MOAs). Therefore, our data show a strong correlation between the activation of NFκB and histologic grade of diffuse gliomas.

Stat3, a known downstream target of the EGFR signaling pathway, is activated in a variety of tumors.[35] Recently, activation of Stat3 in GBMs and LGAs has been reported using EMSA.[44,45] Schaefer *et al*,[44] however, failed to demonstrate significant activation of Stat3 immunohistochemically in glioma tumor cells. To address this inconsistency, we examined the activation of Stat3 in 259 human glioma samples of different grades using a monoclonal antibody that detects the active pStat3. We observed only focal nuclear staining for pStat3 in gliomas. Moreover, the positive rate was less than 9% in high-grade gliomas and was completely negative in all 10 gliosarcoma and 10 GBM cell lines examined. There was no correlation between Stat3 activation and histologic grade. This finding is consistent with the previous observation that failed to detect appreciable amounts of constitutively activated Stat3α in eight untreated GBM cell lines.[46] Therefore, our data do not support the notion that activation of Stat3 plays a significant role in glioma progression.

U251MG cells with high levels of activated Akt also showed high levels of NFκB binding activity (Figure 5). Phosphorylated Akt can activate NFκB either through activation of IKK or by direct phosphorylation of the NFκB p65 subunit.[21–23,26–28] The immunohistochemical data from the present tissue microarray study show a significant correlation between the activation of Akt and the activation of NFκB in diffuse gliomas. This correlation is further supported by Western blot analysis of frozen GBM tissue samples, which showed a 100% concordant expression rate for pAkt and pNFκB p65. Our data are therefore consistent with the notion that activation of Akt may lead to activation of NFκB in diffuse gliomas. The functional importance of activated Akt in NFκB activation in human gliomas is further strengthened by the marked decrease in NFκB-binding activity observed when

U251MG cells are transfected with PTEN or treated with the PI3 kinase inhibitors, wortmannin and LY294002. As expected, U251MG/PTEN cells that express PTEN showed no detectable pAkt and were negative for pAkt by immunohistochemistry (Figure 5). The PTEN-expressing U251MG cell line also showed very low levels of NFκB-binding activity. Furthermore, treatment of U251MG cells with the PI3 kinase inhibitors wortmannin and LY294002 not only reduced Akt activation, but also resulted in a concomitant decrease in NFκB-binding activity. Therefore, our results show that activation of Akt plays a role in the activation of NFκB in gliomas. On the other hand, a significant number of gliomas that were negative for pAkt showed overexpression and activation of NFκB, suggesting that other signaling pathways may also be involved in the activation of NFκB in diffuse gliomas.

The activation of both Akt and NFκB has been associated with antiapoptosis and cell proliferation in GBM cell lines and in animal studies. Activation of Akt and NFκB may lead to tumor resistance to TNF-α, irradiation, or chemotherapy in GBM and other malignant gliomas. Thus, the ability to inhibit Akt and NFκB might confer an increased sensitivity to therapeutic modalities. The finding that activation of Akt and NFκB correlates with histologic grade in diffuse gliomas also offers potential avenues for the development of novel therapeutic strategies.

Finally, results of the present study demonstrate that molecular analyses performed on consecutive tissue microarray sections facilitate direct comparison of alterations of multiple molecular markers in nearly identical, histologically highly conserved tumor regions. The tissue microarray approach is thus well suited for rapid molecular dissection of oncogenetic regulatory pathways and subsequent correlation with histologic grade, tumor progression, and various important clinical characteristics.[47]

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