mTOR, S6 and AKT Expression in Relation to Proliferation and Apoptosis/Autophagy in Glioma

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Abstract. *Background: The mammalian target of rapamycin (mTOR) controls cell growth through protein synthesis regulation. It can be activated by protein kinase B (AKT) or through ribosomal S6 kinase (S6K1). In malignant glioma, mTOR inhibitors have antiproliferative and proapoptotic effects and mTOR has been suggested as a target of therapies, thus it is worthwhile to verify its relations with the phosphatidylinositol-3-kinase (PI3)/AKT cascade, proliferation and apoptosis in human gliomas. Materials and Methods: In a series of 64 gliomas, including high- and low-grade tumors, AKT, mTOR, S6, caspase-3, poly(ADP-ribose) polymerase 1 (PARP1) and cleaved PARP1, Ki-67/MIB.1 and beclin 1 were studied by molecular biology techniques, quantitative immunohistochemistry and Western blotting. Results: mTOR (phospho-mTOR), S6 (phospho-S6), AKT (phospho-AKT) levels and Ki-67/MIB.1 labelling index (LI) increased with increasing grade of malignancy. Epithelial growth factor receptor (EGFR) amplification correlated with EGFRwt and EGFRvIII immunohistochemistry, and with AKT expression; the latter correlated with mTOR expression, whereas S6 expression correlated with Ki-67/MIB.1 LI. Within the category of glioblastoma, S6 but not mTOR correlated with proliferation. mTOR did not show correlation with apoptosis, whereas it was inversely correlated with beclin 1, in line with the observation that autophagy is not activated in many malignancies. Conclusion: The relationship of S6 with the proliferation markers emphasizes the importance of the position of S6K1 downstream AKT in the PI3/AKT pathway.*

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The mammalian target of rapamycin (mTOR) is a serine/threonine (Ser/Thr) protein kinase, a member of the phosphatidylinositol-3-kinase-related kinase (PKK) family (1). It controls cell growth through protein synthesis regulation by integrating signals from growth factors (2). mTOR can be activated by AKT directly or indirectly, through tuberous sclerosis complex (TSC1 and 2) (3, 4) by guanosine triphosphatase (GTPase) Rheb inhibition. Its downstream effectors are eukaryotic initiation factor 4E and the ribosomal S6 kinase (S6K1). By the first mechanism, 4E-BP1 suppressor protein factor is phosphorylated and inactivated, whereas by the second mechanism, translation of mRNA by S6 effector of S6K1 is enhanced (5). S6K1, as a key regulator of mRNA translation, plays an important role in cell cycle progression through the G_1 phase of proliferating cells and in the synaptic plasticity of terminally differentiated neurons (6, 7). Activation of S6K1 involves the phosphorylation of its multiple Ser/Thr residues (8). S6 is the most studied effector of S6K1 (9).

The activation of mTOR is realized by AKT, but it has been demonstrated that this may also happen through S6K1, the activation of which is mediated by TSC (10, 11). The latter can also activate signal transducer and activator of transcription-3 (STAT3) (12). Moreover, in glioblastoma, there is a close correlation between AKT and TSC2. Importantly, AKT activation has been found to be more closely related to S6K1 and S6 than to mTOR, suggesting a downstream effect of AKT through TSC2 and S6 kinase (13).

mTOR inhibition has anticancer effects. In two glioblastoma cell lines, rapamycin (an mTOR inhibitor), and EKI 785 an epithelial growth factor receptor (EGFR) inhibitor, showed antiproliferative and proapoptotic synergistic effects (14). Furthermore, rapamycin induced apoptosis in two cell lines of rhabdomyosarcoma (15). Apoptosis is induced through the inactivation of Bcl-2 antagonist of cell death (BAD) (16).

mTOR may have a pleiotropic function in the regulation of cell death (17) because it is also involved in autophagy (18). This type of cell death can be induced by the inhibition of mTOR because under normal conditions, mTOR inhibits this cell death through autophagy genes (ATG) (19).

Table I. *Evaluation score system.*

Marker	Category		
	A	B	C
Phospho-AKT,	[1]Diffuse, <20%	[1]Diffuse, 20-50%	[2]Diffuse, >50% or >20%
Phospho-mTOR	"	"	"
Phospho-S6	"	"	"
Ki-67/MIB.1	<20%	20-30%	>30%

[1]With or without foci; [2]with multiple foci.

Autophagy is a caspase-independent process of degradation, with the formation of autophagosomes and their fusion with lysosomes (20), regulated not only by mTOR and its complexes with ATG but also by beclin 1 or ATG6. Beclin 1 is one component of a complex that includes the class III PI3K (PIK3C3; also known as Vps34), which is stimulatory for autophagy (21). Recently, an AKT inhibitor showed anticancer and radiosensitizing effects on U87-MG and U87-MG-δ−EGFR cells by inducing autophagy and abolishing radioresistance (22). AKT inhibitor, therefore, may represent a promising new therapy as a single treatment, or in combination with radiation for malignant glioma, including radioresistant gliomas expressing δ-EGFR. Moreover, telomere 3' overhang-specific DNA oligonucleotides (T-oligos) inhibit mTOR and STAT3. Their inhibitors, rapamycin and AG490, respectively, sensitize malignant gliomas cells by augmenting autophagy (23, 24). Other observations are available on the efficacy of mTOR inhibitors (also in association with other procedures), in countering malignant cell growth (24-29).

Poly(ADP-ribose) polymerase 1 (PARP-1) is a nuclear protein that intervenes in many processes including DNA repair. Upon DNA damage, PARP-1-binding domain binds to DNA strand breaks, synthesis of poly(ADP-ribose) takes place and NAD^+ is consumed. PARP-1 functions as a switch between cell survival with DNA repair and induction of cell death, because of ATP depletion and because it is cleaved by caspases otherwise activated. Its activation can denounce apoptosis in progress (30, 31).

In a series of 64 gliomas, the correlations of mTOR, AKT and S6 expression with histological grade, cell proliferation and apoptosis/autophagy were studied.

Materials and Methods

Surgical samples were collected from the Department of Neuroscience, University of Turin and from the Clinical and Experimental Medical Department of East Piedmont, University of Novara. Sixty-four gliomas were studied: 34 glioblastomas (GBMs), 10 grade III anaplastic astrocytomas, 10 grade II astrocytomas and 10 oligodendrogliomas (5 grade II and 5 grade III), diagnosed according to the WHO. Surgical samples were fixed in buffered formalin, embedded in paraffin and cut into 5 μm-thick serial sections.

Table II. *Immunohistochemical frequencies in the three glioma types.*

Activated pathway	Astrocytoma (n=20)		GBM (n=34)	Oligodendroglioma (n=10)	
	Grade II	Grade III	Grade IV	Grade II	Grade III
Phospho-AKT	0%	50%	56.2%	0%	20%
Phospho-mTOR	0%	70%	81.8%	0%	20%
Phospho-S6	0%	30%	82.3%	0%	0%

Immunohistochemistry (IHC). The following primary antibodies were used: rabbit polyclonal anti-phospho-mTOR (Ser2448 (#2971); diluted 1:75), mouse monoclonal anti-phospho-AKT (Ser473; (#4051) diluted 1:100), rabbit polyclonal anti-phospho-S6 (Ser240/244 (#2215); diluted 1:100), rabbit polyclonal anti-PARP1 (#9542, diluted 1:200), rabbit polyclonal anti-cleaved PARP1 (Asp214) (#9541, diluted 1:50) all from Cell Signaling Technology, Beverly, MA, USA; rabbit polyclonal anti-beclin 1 (sc-11417; diluted 1:200 Santa Cruz Biotechnology, Santa Cruz, CA, USA); mouse monoclonal anti-Ki-67/MIB.1 (M7240; diluted 1:100 Dako, Carpinteria, CA, USA); rabbit polyclonal anti-caspase-3 (AB3623; diluted 1:20 Chemicon International Inc., Temecula, CA, USA). The immunohistochemical reactions were carried out on consecutive sections with StreptABC complex/HRP (Dako) and diaminobenzidine (DAB) (Roche Diagnostics, GmbH, Penzberg, Germany). Microwave antigen retrieval was performed with 0.01 M citrate buffer (pH 6.0 or 7.4) (3×3 min at 600 W). Negative controls were performed by omission of the primary antibody and positive ones for phospho-mTOR, phospho-AKT and phospho-S6 with human breast cancer and for caspase-3, PARP1 and cleaved PARP1 with malignant neuroblastoma.

Evaluation of immunohistochemical staining. In all of the samples, all proliferating areas were studied with the exclusion of necrotic or regressive ones. Expression was evaluated according to intensity (–, +, ++), frequency of positive nuclei/cells (<20%, 20%-50%, >50%) and distribution (focal or diffuse) with a score system including both types of expression and three categories A, B, and C (Table I).

The labelling index (LI) was calculated as the mean of the area with the highest frequency of positive nuclei/cytoplasms by visual analysis, containing at least 1,000 cells. Usually the areas were of 5 high power fields (HPF) with immersion oil

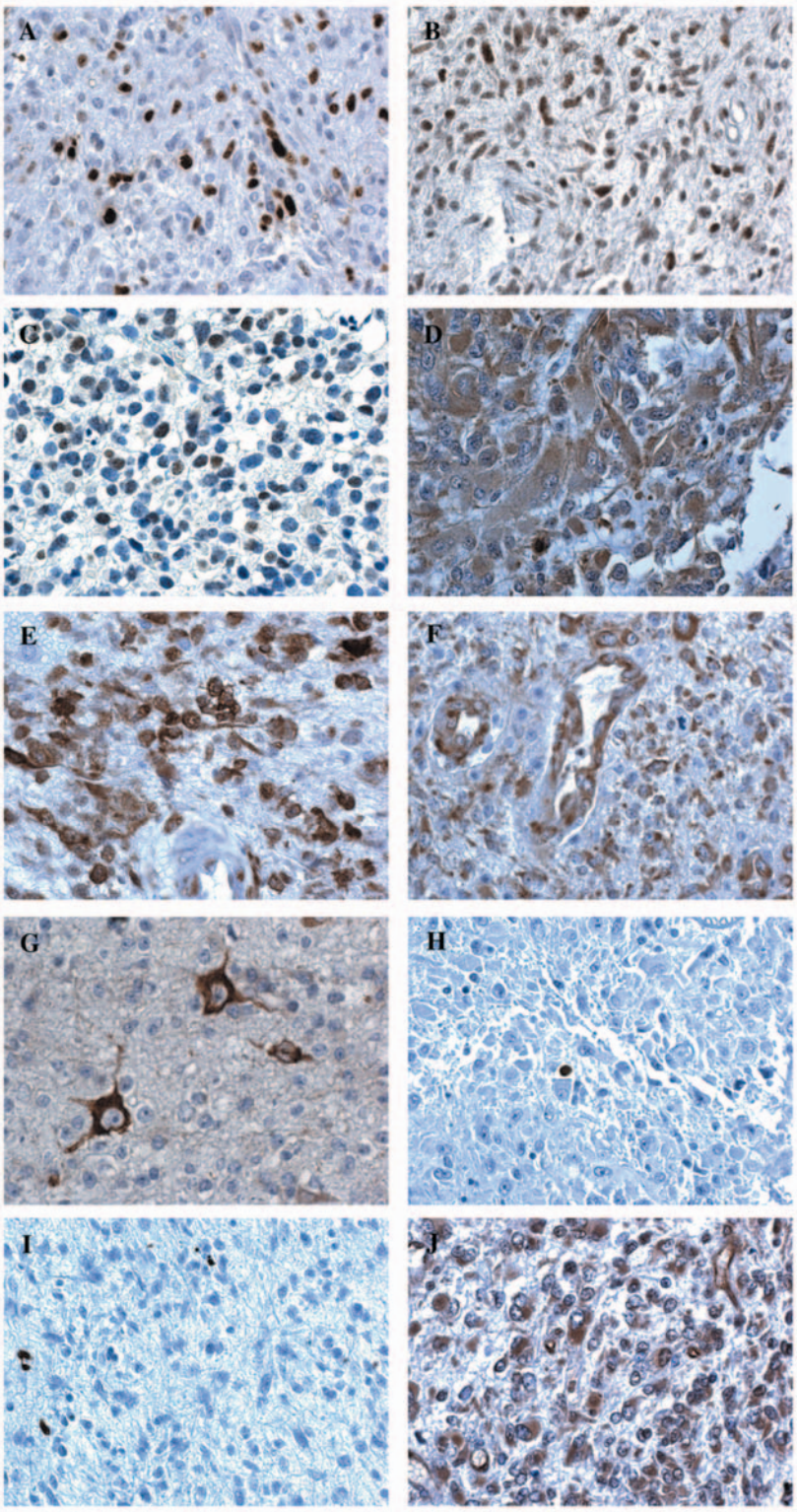

Figure 1. *Immunohistochemistry of glioma. A, A high number of Ki-67/MIB.1-positive nuclei in GBM; B, positive nuclear staining for mTOR in GBM; C, positive nuclear staining for AKT in oligodendroglioma; D, positive cytoplasmic staining for AKT in GBM; E, positive cytoplasmic staining for pS6 in GBM; F, positive staining in endothelial cells for pS6 in GBM; G, positive cytoplasmic staining of neurons in peritumoral cortex for pS6 in GBM; H, caspase-3 positive nucleus in GBM; I, cleaved-PARP-positive nuclei in GBM; J, beclin 1-positive cytoplasmic and nuclear staining in GBM (×400, DAB).*

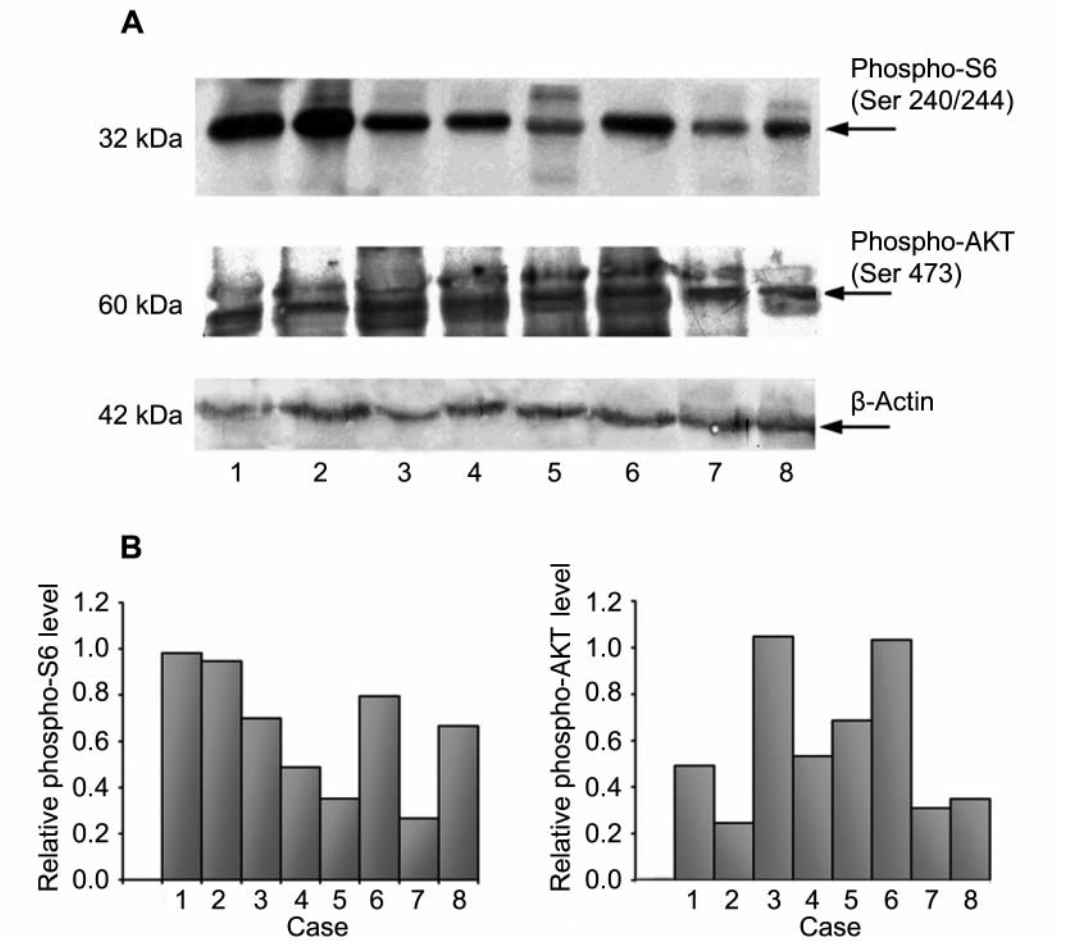

Figure 2. *A, Western blotting of phospho-AKT and phospho-S6 expression. Positive bands in samples from 8 cases of GBM. B, Quantitative analysis of the phospho-S6 and phospho-AKT levels normalized to β-actin data.*

corresponding to 0.001 mm^2. For GBMs only, caspase-3 and cleaved-PARP1 expressions were calculated as the percentage of positive nuclei/cytoplasms after counting the entire section or at least 1,000 cells. In addition, in GBMs only, Ki-67/MIB.1 LI was also calculated in areas with maximum phospho-S6 LI and *vice versa*.

Protein extraction and Western blotting analysis. Paraffin sections for protein extraction were deparaffinized and homogenized in a RIPA buffer with a protease and phosphatase inhibitor cocktail (Sigma Aldrich Co., St. Louis, MO, USA). Total protein of tissue lysates was quantified by BCA™ Protein Assay Kit (Pierce Biotechnology, Rockford, IL, USA) and equal amounts of protein (100 μg) were resolved by SDS-PAGE with 12% gel and transferred to nitrocellulose membranes (Biorad, Hercules, CA, USA). Blots were incubated overnight at 4˚C with rabbit monoclonal anti-phospho-AKT (Ser473), diluted 1:1000 (#3787; Cell Signaling Technology), and for phospho-S6 with the same antibody as in IHC, and then with the appropriate horseradish peroxidase (HRP)-conjugated secondary antibodies (Dako). Protein signals were detected using the ECL Detection System (GE Healthcare, Buckinghamshire, UK). A specific anti-β-actin antibody (A5441; Sigma Aldrich Co.) was used for normalizing sample loading and transfer. Bands intensity was quantified by densitometry using NIH Image J (RSB, NIMH, Bethesda, MD, USA).

Statistical analysis. Associations between categorical variables were evaluated with 2×2 contingency tables by two-tailed Fisher's exact test. Correlation analyses were performed using Pearson's correlation coefficient. Survival analysis was carried out by the Kaplan-Meier method (SPSS version 15.0, Chicago, IL, USA).

Results

Ki-67/MIB.1. The LI was 5% (2-6%) for grade II astrocytomas, 12% (5-20%) for grade III astrocytomas, 2% (0-10%) for grade II oligodendrogliomas, 15% (12-28%) for grade III oligodendrogliomas and 23% (12-30%) for GBMs (Figure 1A).

Phospho-mTOR immunohistochemistry. The staining was nuclear with a score of A in grade II astrocytomas and oligodendrogliomas, B in grade III astrocytomas and oligodendrogliomas, and C in GBMs (Figure 1B). The frequency values were 0% , 70% , and 81.8% for grade II and III astrocytomas and GBMs, respectively. In oligodendrogliomas, the values were 0% and 20% for grade II and III, respectively.

Phospho-AKT immunohistochemistry. In grade II astrocytomas and oligodendrogliomas, the staining was rather nuclear with a score of A (Figure 1C). In grade III astrocytomas and oligodendrogliomas, the staining was still nuclear with a score of B. In GBMs, the staining was mainly cytoplasmic with a score of C and was only occasionally nuclear (Figure 1D). In GBMs, microvascular proliferations were negative; occasionally, the staining was more intense in cells around vessels and outside pseudopalisades. The frequency values were 0% , 20% , and 80% for grade II and III astrocytomas and GBMs, respectively. In oligodendrogliomas, the values were 0% and 20% for grade II and III, respectively.

Phospho-S6 immunohistochemistry. The staining was prevailingly nuclear in low-grade gliomas and cytoplasmic in GBMs. The score was A in grade II gliomas, B in grade III gliomas and C in GBMs (Figure 1E). Endothelial nuclei and those of microvascular proliferations were positively stained (Figure 1F). Moreover, neurons of the peri-tumoral cortex and those entrapped in the tumors were intensely positive (Figure 1G). The frequency values were 0% , 30% , and 82.3% for grade II and III astrocytomas, and GBMs, respectively. In oligodendrogliomas, the values were 0% and 0% for grade II and III, respectively.

Caspase-3, PARP1, and cleaved PARP1 immunohistochemistry (GBMs only). Caspase-3 was positive as cytoplasmic or nuclear staining or in apoptotic bodies (Figure 1H). PARP1 staining, was positive in all of the nuclei of the tumor. Nuclei were occasionally positive for cleaved-PARP1, which was distributed like caspase-3: very rarely in proliferating areas and more abundant in perinecrotic palisades (Figure 1I). The latter were not considered in the present study. Perinecrotic areas, which were not counted in this work, contained many more positive nuclei. The percentage of positive cells/nuclei was constantly <0.02.

Beclin 1 immunohistochemistry. The study was limited to GBMs. Positive, irregular, both nuclear and cytoplasmic staining was found in two GBMs only (Figure 1J).

Western blotting (GBMs only). Western blotting analysis showed positive bands for phospho-AKT and phospho-S6, showing the same variability as immunohistochemistry (Figure 2A, B).

Correlation analysis. Phospho-AKT, phospho-S6, phospho-mTOR, and Ki-67/MIB.1 appeared to be strongly related to histological malignancy (Table II), but no correlation could be found with survival within the category of GBMs. Previously, we reported a significative correlation of wild-type (wt) EGFR immunohistochemistry with phospho-AKT levels by Western blotting analysis and of the latter with phospho-AKT immunohistochemistry (32). In the present study, phospho-S6 immunohistochemistry showed a positive correlation with phospho-S6 levels by Western blotting analysis (Pearson's correlation coefficient r=0.566, p=0.0014).

In a study of the same material, no correlation was found between EGFR, neither by amplification nor immunohistochemistry (32) and presently between phospho-mTOR and phospho-S6. The only significant correlation was that between phospho-AKT and phospho-mTOR immunohistochemistry (p=0.0128). There was linear correlation between Ki-67/MIB.1 and phospho-S6 LI comparing the peaks of frequency (average 30 areas) (Pearson's correlation coefficient r=0.487, p=0.0063). Caspase-3 and cleaved-PARP1 LI were very low and the percentage of positive cells/nuclei was constantly <0.02; no correlation study was possible with the other antigens studied. In GBMs, no correlation of phospho-mTOR and phospho-S6 levels, and Ki-67/MIB.1 LIs was found with survival by the Kaplan-Meier method. Beclin 1 was inversely correlated with phospho-mTOR, phospho-S6, and Ki-67/MIB.1 LIs.

Discussion

The frequency of phosphorylated mTOR, AKT, and S6 expression was found to increase with the histological grade in astrocytic and oligodendrocytic tumors. AKT exhibited a different expression pattern in the different grades: cytoplasmic in high-grade and nuclear in low-grade gliomas, including oligodendrogliomas in which the frequency only slightly increased with anaplasia. In GBMs, the prevailing diffuse cytoplasmic staining was consistent with other observations (33); however, it was also found to be either nuclear or cytoplasmic (34). There is an abrupt increase of AKT expression in GBM with respect to other diffuse astrocytomas, and the double localization of AKT corresponds to its regulation of the cell cycle from both compartments (35). The prevailing nuclear localization in low-grade gliomas, where AKT expression is infrequent, could mean that the regulatory mechanism is different in comparison with tumors with cytoplasmic localization.

In our material, it was previously observed that EGFR amplification correlated with immunohistochemistry, EGFRvIII, and AKT (32), and this finding was consistent with the activation of the PI3/AKT pathway by growth

factors (*i.e.* EGFR) (36). There was a positive correlation between EGFRwt immunohistochemistry and AKT Western blotting. In the present series, the only positive correlation was that between AKT and mTOR. mTOR, AKT and S6 increased with histological grade, reaching maximum expression in GBM, but, within this tumor category, positivity did not correlate either with survival or with Ki-67/MIB.1 LI, with the exception of S6. This correlation concerns only the peaks of the highest frequency. The regional heterogeneity of GBM accounts for the lack of correlation of the aforementioned antigen expressions. On the other hand, the existence of morphological prognostic factors in GBM is still a matter of discussion: if the two variants, namely gigantocellular GBM and gliosarcoma, and tumors with oligodendroglial areas are not considered, no phenotypic feature has been recognized as being of prognostic value. Proliferation markers fall into this category of factors: in spite of some demonstrations to the contrary most of them cannot be used as markers of prognosis (37).

The correlation of S6 with Ki-67/MIB.1 may have some importance in the understanding of what happens in the PI3/AKT pathway downstream of AKT, which is still a matter of discussion. It has been demonstrated that S6K1, but not AKT, directly phosphorylates mTOR. When S6K1 is knocked-down with inhibitory RNA, phosphorylation of mTOR is reduced, despite elevated AKT activity (11, 13). On the other hand, in GBM it has also been demonstrated that there is a close correlation between AKT and S6 activation (13). We did not find such a correlation, but that between S6 and Ki-67/MIB.1 could be in line with this observation. On the contrary, a correlation of AKT and mTOR was found, even though with contrasting meaning. The extreme heterogeneity of reaction for all of these antigens and the difficulty of the quantitative evaluation of their expression associated with the low number of cases examined could account for the discrepancy. The conclusion that downstream AKT effects are primarily mediated by S6K1, the activity of which is to enhance proliferation rather than to inhibit apoptosis, could be in line with our results. It must be considered that in our previous work, a correlation between AKT and STAT3 was found in the same cases (32). The activation of STAT3 by AKT may be the intermediate step between EGFR aberration and interleukin (IL)-6 dysfunction and STAT3 activation (37) acting through the Bcl-2 family of antiapoptotic proteins (38).

No inverse correlation was found between the antigens studied and apoptosis, as revealed by our assays for caspase-3 and cleaved PARP1, the findings of which coincide, in spite of the inhibition of apoptosis by mTOR, S6 and AKT. Technical reasons may account for the lack of correlation. Only proliferating areas have been considered in our work, whereas apoptosis in GBM is mainly represented in perinecrotic areas. In the former, theoretically, apoptosis should be triggered mainly by the transcriptional or intrinsic pathway and in the latter by the extrinsic or receptorial one (39). To find out which of these pathways is active might be uninformative because the apoptotic signalling that comes down from death receptors can cross to the transcriptional pathway through BH3-interacting domain death agonist (BID) (40). It seems, however, that this is unlikely in GBM, because of the low detectable levels of caspase-8 in gliomas (41). Another factor explaining the lack of an inverse correlation, as was found previously with STAT3, is the low number of apoptotic nuclei in proliferating areas of glioblastoma. It must be remarked that in low-grade gliomas where apoptosis is rather rare, mTOR and STAT3 are barely expressed.

The positive staining of S6 in endothelial cells and in microvascular proliferations may reflect the propensity for proliferation of these cells in GBM, and it must be of some meaning that they are not at the same time positive for mTOR. The positive staining of neuronal cytoplasm can be explained by its intervention in the synaptic plasticity of terminally differentiated neurons (6, 7).

Finally, the near lack of expression of beclin 1 in GBM was expected. It is in line with what is known from the literature on the relationship between autophagy and cancer (20). Beclin 1 may contribute to cancer progression because it enhances cancer cell survival, but it may also show the opposite effect because of degradation of mitochondria, which can release death proteins or eliminate sources of free radicals (42). Malignant cells have lower protein catabolic activity and show low autophagic activity (43). In brain tumors, beclin 1 decreases with malignancy and in GBM its expression is rather nuclear, indicating a loss of gene function (44). Our findings of a practical lack of beclin 1 positivity in GBM are in line with these observations and are also consistent with the high expression of mTOR. In this regard, the induction of autophagy by inhibitors of mTOR (45) and by radiotherapy and chemotherapy, in particular with temozolomide (46-48), is of great significance because it indicates the steps in the pathway to autophagy as a target for therapies.

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