Could changes in the regulation of the PI3K/PKB/Akt signaling pathway and cell cycle be involved in astrocytic tumor pathogenesis and progression?

A. HLOBILKOVA[1*], J. EHRMANN[1], E. SEDLAKOVA[1], V. KREJCI[1], P. KNIZETOVA[1], M. FIURASKOVA[1], M. KALA[2], O. KALITA[2], Z. KOLAR[1]

[1]Institute of Pathology and Laboratory of Molecular Pathology, Faculty of Medicine, Palacky University, Hnevotinska 3, Olomouc, CZ-775 15, Czech Republic, e-mail: hlob@tunw.upol.cz, [2]Clinic of Neurosurgery, Teaching Hospital, I. P. Pavlova 6, CZ-775 20, Olomouc

Received January 1, 2007

The most frequent alterations found in astrocytomas are two major groups of signaling proteins: the cell cycle and the growth factor-regulated signaling pathways. The aim of our study was to detect changes in expression of the following proteins: the tumor suppressors PTEN, p53, and $p21^{Waf1/Cip1}$, glial fibrillary acidic protein (GFAP, as a marker of astroglial differentiation), the phosphorylated form of protein kinase B/Akt (PKB/Akt), which is downstream to the epidermal growth factor receptor (EGFR), and MDM2, which degrades p53. Paraffin-embedded astrocytoma tissue samples from 89 patients were divided into low grade (grade I-II; 42 samples) and high grade astrocytomas (grade III-IV; 47 samples). Mouse monoclonal antibodies against GFAP, PTEN, PKB/Akt phosphorylated on serine 473, EGFR, p53, $p21^{Waf1/Cip1}$ and MDM2 were used, followed by standard indirect immunohistochemical method. EGFR protein was detected in 29 % of low grade and in 60 % of high grade astrocytomas. The expression of phosphorylated PKB/Akt was found in roughly the same proportions: in 86% of low grade and in 79% of high grade astrocytomas. PTEN was not found in most of astrocytomas, 64% of low grade and 74% of high grade tumors showed no PTEN staining. Overexpression of the mutated form of p53 or loss of p53 expression, however, was found in about 63% in both groups of astrocytomas with no differences between them. GFAP expression was decreased in tumor astrocytes compared to normal astrocytes and this decreased with grading. GFAP positive tumor cells were detected in only 50% of low grade, and 32% of high grade astrocytomas. The level of MDM2 expression was similar in both grades. Loss of $p21^{Waf1/Cip1}$ expression was shown in 20% of low and in 45% of high grade tumors. In the subgroup of high grade tumors with wild type p53, 86% showed $p21^{Waf1/Cip1}$ expression, whereas in the subgroup of high grade tumors with altered p53, only 35% displayed $p21^{Waf1/Cip1}$. We conclude that EGFR expression increases with astrocytoma grading. EGFR activation may subsequently lead to stimulation of the PKB/Akt survival pathway. PTEN defects may also participate in aggressive tumor behaviour through activation of the PKB/Akt pathway. The alteration of p53 supports the finding that the cell cycle regulation is also disrupted during development of astrocytomas. The changes in PTEN and p53 expression, and activation of PKB/Akt are events in the early stages of astrocytomagenesis. EGFR is one of the factors, which drives the progression of astrocytomas from low to high grade stage.

Key words: astrocytoma, PI3K/PKB/Akt pathway, cell cycle and immunohistochemistry

Astrocytomas are the most common form of primary brain tumor and prognosis for patients with high-grade astrocytoma is poor, despite intense efforts to develop new treatment approaches. The World Health Organisation (WHO) classifies astrocytomas into four grades, with grades I and II presenting as benign tumor and grades III and IV displaying more malignant phenotypes [1]. Grade I is represented by pilocytic astrocytoma, which is a circumscribed, slowly growing often pseudocystic tumor found in children and adolescents. Grade II – diffuse astrocytomas, typically occurring in young adults, is a well-differentiated tumor, which diffusely infiltrates the brain. Astrocytomas of grade II invariably progress to higher grade tumors over the course of few years up to a decade. Fibrillary, gemistocytic and protoplasmic are histologic variants of diffuse astrocytoma. Grade III – anaplastic astrocytoma is a recurrent tumor that has progressed from diffuse astrocytoma and displayes increased cellularity, atypical nuclei,

*Corresponding author

mitosis and blood brain barrier abnormalities. Grade IV – multiform glioblastoma is the most malignant poorly differentiated astroglial neoplasm found in older adult patients. This tumor is highly heterogenous and displays cellular polymorphy, atypical nuclei with palisading, high mitotic activity, microvascular proliferation, blood vessel thrombosis and necrosis. Glioblastoma is manifested de novo as primary glioblastoma or as secondary glioblastoma – based on progression from diffuse or anaplastic astrocytoma. It remains unclear whether astrocytomas arise from the dedifferentiation of mature astrocytes or by the transformation of neural stem cells or early glial progenitors [1]. Although these cells are localised in subventricular zone [2] and hippocampus [3], which is not typical for glioma development, the proliferatory and migratory capabilities of these cells predict that tumor formation could occur anywhere in the brain [1]. These premalignant aberrantly located precursor cells, could go on to acquire genetic alterations leading to fully transformed cells. Studies of astrocytomas have led to the identification of two major groups of signaling proteins, whose abnormalities contribute to gliomagenesis: the cell cycle pathway and the growth factor-regulated signaling pathways.

Glial fibrillary acidic protein (GFAP) is an intracytoplasmic filamentous protein which is a part of the cytoskeleton of mature astrocytes in the central nervous system. GFAP gives structural stability to astrocytic processes and is important for astrocyte motility and shape [4]. It has been shown that GFAP is a standard marker specific for cells of astrocytic origin and reduced GFAP expression has been described along with an increase in astrocytoma malignancy potential [4]. The tumor suppressor p53 plays a large role in response to DNA damage and other genomic aberrations. Activation of p53 can lead to cell cycle arrest and DNA repair, inhibits the activity of cdc2 via the p53-induced inhibitor of cyclin dependent kinase $p21^{Waf1/Cip1}$ [5] or triggers apoptosis via a number of distinct mechanisms. p53 also activates the transcription of the PTEN and PKB regulated gene – tuberous sclerosis 2 (TSC2), and therefore functions as a negative regulator of the PI3K signaling pathway [6, 7]. p53 eliminates the PI3K mediated survival and proliferation signal, providing an additional level of protection against continued DNA replication in the presence of genotoxic stress [8]. p53 is the one of the most commonly mutated genes in all types of cancer. Probably the most important modulator of p53 is one of its downstream targeted genes, murine double minute 2 (MDM2), which inhibits p53 transcriptional activity directly by binding to the N-terminal transactivation domain and also functions as an E_3 ubiquitin ligase to serve p53 for proteosomal degradation [9]. Transcription of the MDM2 gene is induced by p53, creating a negative feed-back loop that regulates the activity of the p53 protein and MDM2 expression [10]. $p21^{Waf1/Cip1}$ is a p53 regulated protein with tumor suppressor function, which assembles a complex with cdk2 to inhibit kinase activity and block progression through the G_1/S phases of the cell cycle. Occasionally, it also complexes with cdk4 and 6 [11]. $p21^{Waf1/Cip1}$

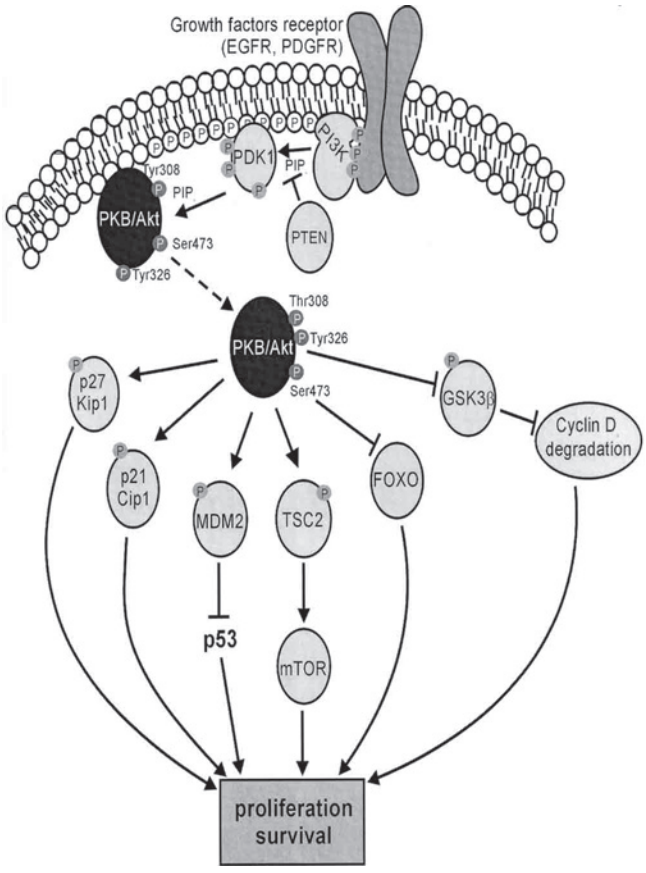

Figure 1. PI3K/PKB/Akt signaling pathway (adapted from Cell Signaling). The phosphatidylinositol-3-kinase (PI3K) is activated by growth receptors: epidermal growth factor and platelet derived growth factor receptors (EGFR, PDGFR). PI3K phosphorylates and converts phosphatidylinositol-(4, 5)-biphosphate (PIP_2) into phosphatidylinositol-(3, 4, 5)-triphosphate (PIP_3), which activates phosphatidylinositol-dependent kinase 1 (PDK1). PTEN negatively regulates PI3K by dephoshorylating of PIP_3 and converting back to PIP_2. PDK1 further phosphorylates and activates protein kinase B (PKB/Akt). Phosphorylated PKB/Akt negatively regulates cyclin-dependent kinase inhibitors $p27^{Kip1}$ and $p21^{Waf1/Cip1}$; enhances activity of MDM2 leading to p53 degradation; prevents GSK3β phosphorylation and degradation of cyclin D_1; inhibits forkhead (FOXO) transcription factors (which mediates apoptosis and cell cycle arrest); phosphorylates and inactivates TSC2 (tuberous sclerosis 2) leading subsequently to activation of mTOR kinase (targeted of rapamycin). Influence of PKB/Akt on targeted substrates results in proliferation and cell survival. → direct stimulatory modification, —| direct inhibitory modification.

can bind and inhibit PCNA, a subunit of DNA polymerase and it may coordinate DNA replication with cell cycle progression [12]. Abnormalities in several receptor tyrosine kinase (RTK) pathways have been implicated in the development of astrocytomas, including: epidermal growth factor, platelet derived growth factor and fibroblast growth factor receptors (EGFR, PDGFR, and FGFR). RTK activates of downstream signaling pathways, such as phosphatidyl-inositol-3 kinase/protein kinase B (PI3K/PKB), Ras/mitogen activated protein

kinase (MAPK), and phospholipase-C-γ/protein kinase C (PLCγ/PKC) pathway [13, 14]. Abnormalities in EGFR are a major contributing factor in glial transformation [15]. One frequent genetic alteration in glioblastomas is deletion of part or the whole chromosome 10. This loss is found in about 60-95% of glioblastomas. PTEN (phosphatase and tensin homologue deleted on chromosome 10) is a tumor suppressor located on chromosome 10 with phosphatase activity that negatively regulates the PI3K pathway [16]. When PTEN is mutated or otherwise inactivated, PI3K effectors are activated – particularly of key survival Ser/Thr kinase PKB and tumorigenesis can be initiated in the absence of any endogenous stimulus. The effect of PTEN can manifest as G_1 cell cycle block [17], induction of apoptosis [18] and/or regulation of migration and invasion [19]. The result of Ser/Thr kinase PKB/Akt activation is apoptosis blockage and increased proliferation. Several direct substrates of PKB/Akt phosphorylation have crucial roles in cell cycle regulation, proliferation and survival. These substrates include $p27^{Kip1}$, $p21^{Waf1/Cip1}$, MDM2, glycogen synthase kinase 3 (GSK3), the forkhead box transcription factors (FOXO) and tuberous sclerosis complex 2 (TSC2), which activates TOR kinase [20, 21] (Fig 1). Aim of our study was to detect the expression of proteins involved in cell cycle machinery as are: p53, $p21^{Waf1/Cip1}$, MDM2, and the growth factor signaling pathway: EGFR, phosphorylated form of PKB/Akt and PTEN, which are implicated in gliomagenesis, and the cytoskeletal protein GFAP, as a marker of astroglial differentiation. Second, to compare these findings in low and high grade astrocytomas.

Materials and methods

Tissue samples. The histological brain astroglioma material, comprising 42 low grade (G I-II) and 47 high grade (G III-IV) specimen, was routinely formalin-fixed and paraffin-embedded. Samples were taken from the archival collection of the Institute of Pathology over the period 1986 – 2004. The patients with low grade astrocytomas ranged from 15 to 80 years old, Md= 48 years and with high grade astrocytomas from 28 to 75 years, Md=52 years. In the group of low grade astrocytomas the gender ratio was (52% females and 48% males. 30 samples of low grade astrocytomas were diagnosed as fibrillary astrocytoma (G II), 2 as gemistocytic astrocytoma (G II), 4 as pilocytic astrocytoma (G I), and 6 samples had a minor oligodendroglial component (G II). In the group of high grade astrocytomas males predominated with 72%. Five samples were diagnosed as anaplastic astrocytoma (G III), 34 as primary glioblastoma (G IV), 6 samples as secondary glioblastoma (G IV) and 2 samples showed minor oligodendroglial component (G IV). All cases were diagnosed according to standard diagnostic criteria of WHO classification [22] at the Institute of Pathology, Palacky University and Teaching Hospital in Olomouc (Czech Republic).

Immunohistochemistry. One paraffin block having the most representative tumor area of each tumor was selected for immnohistochemistry. A summary of the mouse monoclonal antibodies used (source, clone, concentration, time of incubation and unmasking antigen treatment) is shown in Table 1. The incubation with primary antibody was followed by standard indirect immunohistochemical method with Envision plus kit labelled polymer HRP (Dako, Glostrup, DK). Diaminobenzidine (Fluka, Buchs, Schweiz) was used as chromogenic substrate and tissues were counterstained with hematoxylin (Merk, Darmstadt, Germany). The slides were analyzed by two experienced pathologists.

Expression of PTEN was evaluated as histoscore (HS) in the cytoplasm plus in the nucleus. HS = quantity × intensity of staining. Categories of quantity: 1. 0 – 25%, 2. 26 – 50%, 3. 51 – 75%, 4. 76 – 100% of positive tumor cells. Categories of intensity: 1 weak 2. middle, 3. strong staining intensity. GFAP was evaluated quantitatively, as percentage of protein expression in cytoplasm (using the same categories of quantity as PTEN, differences in intensity of staining in whole sample collection was not observed). Phosphorylation of PKB/Akt on serine 473 was evaluated as HS in the cytoplasm and in the nucleus, expression of EGFR was assessed as HS in cytoplasm and cytoplasmic membrane (both proteins by the same categories of quantity and intensity of staining as PTEN).

Table 1. Summary of mouse monoclonal primary antibodies used (source, clone, concentration, time of incubation and unmasking antigen treatment)

Antibody against to	Clone	Source	Concentration	Time of incubation	Antigen umasking treatment
GFAP	6F2	Dako, Glostrup, DK	1:100	over night	1mM citrate buffer, pH 6, MW 20 min, 98°C
PTEN	26H9	CST, Beverly, MA, USA	1:100	over night	1mM citrate buffer, pH 6, MW 20 min, 100 °C
phospho PKB/Akt serin 473	587F11	CST	1:50	30 min	1mM citrate buffer,pH 6, staining automat (Benchmark, VentanaUSA)
EGFR	31G7	Zymed,San Francisco, CA, USA	1:200	over night	digestion by 0,1% trypsine, 10 min
p53	DO-1	MMCI, Brno, CR	undiluted supernatant	over night	1mM citrate buffer, pH 6, MW 20 min, 98°C
$p21^{Waf1/Cip1}$	DCS-60	CST	1:50	30 min	1mM citrate buffer, pH 6, staining automat
MDM2	SMP14	Santa Cruz Biotechnology Inc, CA, USA	1:50	over night	1mM EDTA, pH 8, MW 30 min, 110 °C

CST – Cell Signaling Technology, MW – microwave generator, EGFR – epidermal growth factor receptor, GFAP – glial fibrillary acidic protein, MMCI – Masaryk Memorial Cancer Institute

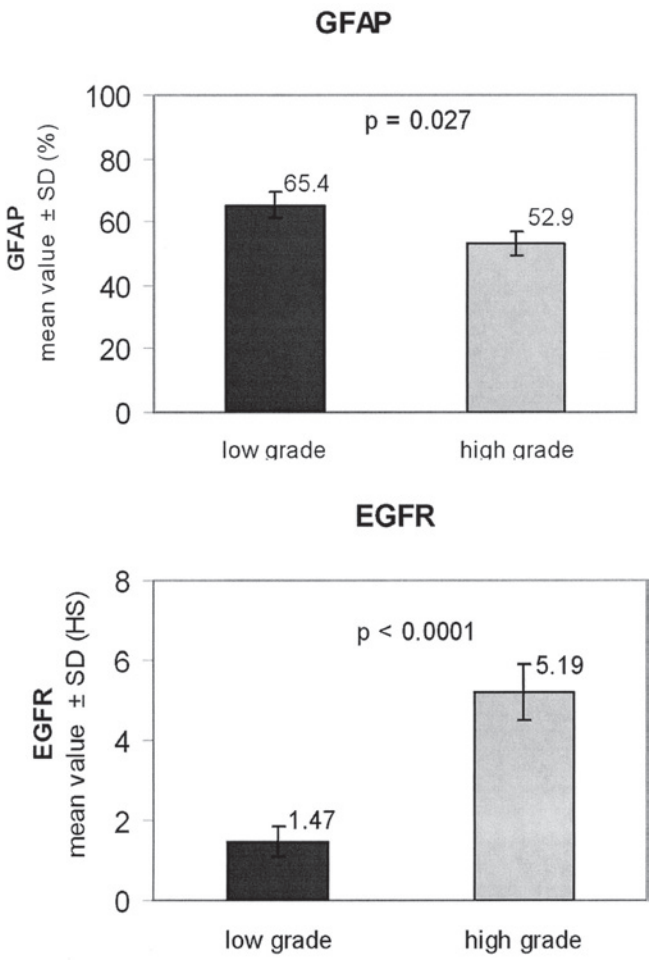

Figure 2. a) The graph shows a mean value of the protein expression of GFAP in percentages with standard deviations (SD) in low and high grade stage of astrocytomas. b) The graph demonstrates the protein expression of EGFR as a mean value of histoscore (HS) with standard deviations in low and high grade stage of astrocytomas.

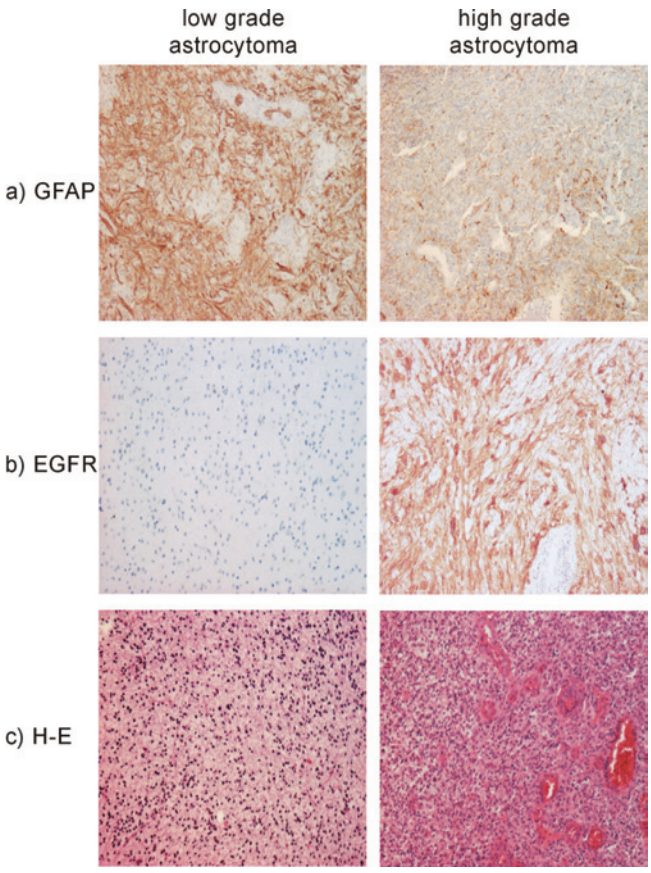

Figure 3. Demonstration of immunohistochemical detection of the protein expression and hematoxylin-eosin (HE) staining of low and high grade astrocytoma. a) GFAP detection shows decrease expression in high grade astrocytoma. For detection was used mouse monoclonal antibody (6F2, Dako). Final magnification was 160×. b) EGFR detection shows high expression in high grade astrocytoma. For detection was used mouse monoclonal antibody (31G7, Zymed). Final magnification was 200×. c) Hematoxylin-eosin staining, final magnification 160×.

p53 expression was evaluated by counting positive nuclei and it was divided into 3 categories: 1. expression in 1% or less than 1% of nuclei – corresponds to WT p53, 2. expression in more than 1% nuclei – corresponds in virtually all cases to overexpression of the mutated form of p53 [23, 24], 3. complete loss of p53 expression. The quantity of $p21^{Waf1/Cip1}$ expression was divided into two categories, 1. negative expression was 0 or almost 0 and 2. positive expression was more than 1%, intensity of expression was evaluated by same categories as PTEN. Expression of MDM2 was assessed as HS in nucleus (by the same categories of quantity and intensity of staining as PTEN). Expression of PTEN, GFAP, EGFR, and p53 was compared in groups of low and high grade tumors, expression of $p21^{Waf1/Cip1}$ and MDM2 was compared in subgroups of low grade tumors with WT or defective p53 and high grade tumors with WT or defective p53.

Photography. Photographs were taken by an Olympus BX50 microscope equipped with Olympus DP50 CCD camera (1392×1047 and 2276×2074 pixel, middle and high-resolution mode).

Statistical analysis. Levene's test for equality of variances, T-test of equality of means and Pearson chi-squere test were used to evaluate the statistical correlations between the groups (low and high grade astroglial tumors and low/high grade tumors with WT or defective p53) and variables. All data were analyzed utilising SPSS 10.1 at the 0,05 level of significance.

Results

There was no significant difference in median age between the group with low grade (48 years) and the group with high grade tumors (52 years). A small subgroup of high grade as-

trocytomas, however, 6 patients with secondary glioblastoma had a median age of 35 years. This suggests a tendency among younger patients to secondary rather than primary glioblastoma (median age 54 years). We found a difference in gender too: in the group of high grade tumors males predominated (72%) compared to females (28%).

GFAP was detected in the cytoplasm only. The category 76% – 100% positive tumor cells was detected in 50% of low grade and in 32% of high grade astrocytomas. The results show that GFAP expression in astroglial tumor cells decreased compare to non-tumor atrocytes. We found a significantly lower expression of GFAP in high grade than in low grade astrocytomas ($p=0.027$; Fig. 2a and Fig. 3a). Reduced GFAP expression in tumor astrocytes is linked to a loss of differentiation. No statistically significant correlations between expression of GFAP and protein expression of PTEN, phosphorylated PKB/Akt, EGFR, GFAP, p53, $p21^{Waf1/Cip1}$ and MDM2 were found.

The tumor suppressor *PTEN* was localised mainly in the cytoplasm but in a few cases it was detected also in the nucleus. The intensity of this protein expression was lower than other detected proteins such as EGFR and phosphorylated PKB/Akt. PTEN expression was found in 30% of all tested astrocytomas on average: in 36% in the group with low grade and in 26% in the group with high grade astrocytomas. It follows from results that PTEN protein was lost in most astrocytomas, in 64% of low grade and 74% high grade. No statistically significant correlations between expression of PTEN and other analysed proteins were found.

The *PKB/Akt* phosphorylated on serine 473 was found in the cytoplasm and in the nucleus. Expression of the phosphorylated form of PKB/Akt was detected in both groups of astrocytomas similarly, in 86% in the group with low grade and in 79% in the group with high grade astrocytomas. Our results showed no significant differences in level of phosphorylation between the groups and no statistically significant correlation between expression of phosphorylated PKB/Akt and other analysed proteins.

EGFR expression was found in the cytoplasmic membrane and also in the cytoplasm. Protein was detected in 29% of low grade and 60% of high grade astrocytomas with stronger intensity in the high grade group. The protein expression of EGFR clearly increases with grade of tumor with a high level of statistical significance $p< 0.0001$. Figure 2b shows the mean value of EGFR as a histoscore (HS for low is 1.47, HS for high grade astrocytomas is 5.19). Figure 3b shows immunohistochemical detection of EGFR in both groups of astrocytomas.

p53 expression was observed in the nucleus only. In our collection, evaluation of p53 expression was done by creating 3 categories: Category 1: expression of wild type (WT) p53, which is characterised by low level of the protein present in few cells (about 1% or less than 1%), Category 2: overexpression of p53, that is, increased expression over the level which characterises WT p53 and which is mostly connected with the mutated form [24]. Category 3: loss of p53 expression which corresponds to mutation of the gene leading to either no expression of protein or expression of unstable and rapidly degradating protein, which is impossible to detect. Alterations to p53, that is, both overexpression of the mutated form and loss of expression, were found in both groups of astrocytomas without marked differences between them (62% in low and 64% in high grade astrocytomas).

Expression of the *$p21^{Waf1/Cip1}$* was found in both nucleus and the cytoplasm in 50% of low grade and in 72% of high grade $p21^{Waf1/Cip1}$ positive tumors. The localisation of $p21^{Waf1/Cip1}$ in the nucleus was only shown by 3% of low grade and 22% of high grade $p21^{Waf1/Cip1}$ positive astrocytomas. The localisation of $p21^{Waf1/Cip1}$ in the cytosplasm only, was observed in 47% of low grade and 6% of high grade $p21^{Waf1/Cip1}$ positive astrocytomas. 80% of low grade astrocytomas expressed $p21^{Waf1/Cip1}$ and with no significant differences between the groups having WT p53 and altered p53 (overexpression of mutated form and loss of protein). In high grade tumors, expression of $p21^{Waf1/Cip1}$ was observed in 55% tumors. In the group of high grade tumors with WT p53, $p21^{Waf1/Cip1}$ expression was found in 86% of tumors. In the group of high grade tumors with altered p53, $p21^{Waf1/Cip1}$ expression was displayed in 35% of tumors only.

Expression of *MDM2* was found in the nucleus only. The average histoscore for MDM2 was 2,7 for low and 3,4 for high grade astrocytomas with very little difference between them. No statistically significant correlations between expression of EGFR, p53, $p21^{Waf1/Cip1}$ and MDM2 proteins were found.

Discussion

In accordance with these findings, a recent population study done in Switzerland showed that primary glioblastomas developed in older patients with a mean age of 62 and secondary glioblastomas in younger patients with a mean age of 45. In the Swiss study, were evaluated also other markers such as the expression of PTEN which was altered in 24%, p53, changed in 31% and EGFR, amplified in 34% of astrocytomas [25].

PTEN mutations are described in a wide spectrum of other sporadic malignancies apart from astrocytomas in advanced stage, including melanoma, carcinoma of prostate, breast, endometrium, kidney and lung. WANG et al [26] showed 44% of primary and secondary high grade astrocytomas with PTEN mutations. FIANO et al [27] published PTEN mutations in 27-40% of glioblastomas. Our results show comparable loss of PTEN staining in both groups of astrocytoma grade. Only 10% of astrocytomas, showed loss of PTEN during transformation from low to high grade stage: in the majority of our cases, loss of PTEN was an event in the initial phase of astrogliomagenesis. FIANO et al [27] found no relationship between EGFR amplification (increased expression) and

PTEN mutations, as our results also indicate. CAI et al [28] described a connection between PTEN and EGFR at the level of glioblastoma cell lines. They introduced PTEN into the U87 glioblastoma cell line and this resulted in inhibition of cell invasion even in the presence of the constitutively active EGFR – with mutation type III. Further, coexpression of EGFR with mutation type III and PTEN by glioblastoma cells was associated with responsiveness to EGFR kinase inhibitor erlotinib [29].

We anticipated that the higher rate of PKB/Akt phosphorylation, in other words, increase in its activity, would positively correlate with grade of astrocytomas. Our results, however, show almost the same level of PKB/Akt phosphorylation in both astrocytoma groups. We conclude that the phosphorylation of PKB/Akt on serin 473 is not a late event in tumorigenesis. Rather activation of this phosphosite probably occurs in the early stages of astrocytoma formation. Activation of PKB/Akt via phosphorylation also takes place at other sites such as threonine 308 and tyrosine 326 and thus could potentially participate in the transition from the low to the high grade stage but can also be phosphorylated in the initiation of tumor development. NAKAMURA et al [30] published an interesting observation on the association between greater PI3K/PKB activity and greater resistance to radiotherapy of glioblastomas. For this reason, analysis of the PI3K/PKB pathway could be very important and its inhibitors may be potentially effective in treatment. Inhibition of PI3K/PKB signaling might also offer treatment to patients who have tumors with p53 mutation: the p53 apoptotic response requires downregulation of the PI3K/PKB pathway through the transcriptional activation of PTEN. And simultaneous inhibition of the PI3K/PKB pathway and activation of apoptosis downstream of p53 can have synergisctic effects [8].

It was published by BENJAMIN et al [31] and KESARI et al [32], that EGFR is amplified in more than 40% and is overexpressed in more than 60% of glioblastomas. Many of the described genetic abnormalities lead to constitutively active EGFR, and do not require the presence of EGF activating ligand. Further, there is also evidence that glioblastomas express the endogenous ligand, EGF and TGFα in addition to EGFR, which is consistent with the existence of an autocrine growth stimulatory loop [33]. Our results show a strong positive relation between EGFR and tumor grade. EGFR activation may thus be a key factor stimulating the transition of low grade astrocytoma to high grade and EGFR overexpression promotes rapid proliferation and angiogenesis of high grade astrocytomas.

LOUIS [34], BOGLER et al [35], and XU et al [36] observed allelic loss of chromosome 17, where is localised p53 and p53 mutations with equal frequency in low grade gliomas, anaplastic astrocytomas, and secondary glioblastomas, suggesting that p53 is an early event in gliomagenesis. LUIS and CAVENEE [37] described in human gliomas mainly p53 missence mutations tergeted residues that are crucial to DNA binding. LOUIS [34] described mutations and inactivation of p53 in at least 33% of astrocytomas. REIFENBERGER et al [23] found mutation associated with p53 accumulation in 77% of primary astrocytomas and p53 immunopositive tumor cell population increased from primary to recurrent tumors. This finding may indicate a clonal expansion of p53 immunopositive, mutant tumor cells, during astrocytoma progression. Perhaps the genomic instability and inhibition of tumor cell entry into apoptosis may be associated with p53 loss and may facilitate the accumulation of additional genetic mutations that promote transformation from low to high grade astrocytomas [38, 1]. However, some studies reported p53 protein acumulation in high compared to low grade gliomas with the suggestion of an additional role for p53 alteration in glioma progression [38-40]. We conclude from our results, which are in accordance with XU et al [36], that the alteration of p53 does not take part in transition from low to high grade astrocytomas. It is an event in the initial phase of astrocytomagenesis. Samples of individual astrocytomas showed quite heterogenous p53 immnureactivity, a finding reported by previous authors. This very likely corresponds with heterogenous histologic structure of these tumors.

In a study by XU et al [36], in 41 astrocytomas higher levels of $p21^{Waf1/Cip1}$ protein expression were seen in higher histological grades. $p21^{Waf1/Cip1}$ expression positively correlated with proliferation index, but was not related to p53 or MDM2 expression. It was suggested that could be p53 independent pathway, which induce $p21^{Waf1/Cip1}$ expression and high expression of $p21^{Waf1/Cip1}$ can be connected with poor prognosis [36]. Some publications refer to the importance of $p21^{Waf1/Cip1}$ localisation. ZHOU et al [41] describe the transfer of $p21^{Waf1/Cip1}$ from the nucleus to the cytoplasm as a critical step for promoting cancer cell survival and activation of PKB/Akt which is associated with $p21^{Waf1/Cip1}$ phosphorylation and cytoplasmic localisation. Our results show coincident nuclear and cytoplasmic expression of $p21^{Waf1/Cip1}$ in 61% of analysed $p21^{Waf1/Cip1}$ positive astrocytomas and preserved nuclear expression in 53% of low grade and 94% of high grade astrocytomas with $p21^{Waf1/Cip1}$ positivity. Further, we found a decrease in $p21^{Waf1/Cip1}$ expression in the group of high grade tumors by about 25% in comparison with low grade tumors. We observed also a decrease in $p21^{Waf1/Cip1}$ expression in the group of high grade astrocytomas with altered p53 in comparison with the group of high grade astrocytomas with WT p53, but these differences were not statistically significant. According to our results, loss of p53 is probably followed by loss of p53 dependent tumor suppressor – $p21^{Waf1/Cip1}$ in advanced stage of astrocytoma progression. Loss of the $p21^{Waf1/Cip1}$ could take part in the transformation from low to high grade stage.

MDM2 is a gene which has been shown to code for a cellular protein that can complex the p53 tumor suppressor gene product and inhibits its function. REIFENBERGER et al [42] reported that MDM2 represents the second most frequently amplified gene after EGFR in high grade astrocytomas. MDM2 amplification and overexpression have been described in 10%

of high grade astrocytomas [42]. We expected higher expression of MDM2 in tumors with alteration of p53, particularly with loss of p53 expression. However, we did not find, as XU et al [36] did not, any association between MDM2, p53, $p21^{Waf1/Cip1}$ expression, and grade.

To recapitulate, EGFR expression increases with astrocytoma grading. This may subsequently lead to activation of the PKB/Akt survival pathway. PTEN defects, may also participate in aggressive tumor behaviour through the loss of phosphatase and cell cycle inhibitory function, which lead also to activation of the PKB/Akt pathway. Observed alteration of p53 supports the finding that the cell cycle regulation is also disrupted during gliomagenesis. We can say, that alteration of PTEN, p53 expression, and phosphorylation (activation) of PKB/Akt are events in the early stages of astrocytomagenesis. EGFR is a pivotal factor which drives the progression of astrocytomas from low to high grade stage.

We thank Mrs. P. Abrahamova, Mrs. J. Stastna, Mrs. A. Lukasova, Mrs. L. Popova and Mr. O. Blaha for their excellent technical assistance with the experiments and documentation. Our special thanks go to Dr. J. Zapletalova for statistical analysis and Dr. B. Vojtesek from the Masaryk Memorial Cancer Institute (Brno, Czech Republic), for providing the antibody against p53.

This work was supported by grants IGA MZ CR NR/7828-3, IGA MZ CR 1A8244-3/2004 and MSM 6198959216.

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