

## Analysis of VEGF, Flt-1, Flk-1, nestin and MMP-9 in relation to astrocytoma pathogenesis and progression.

A. HLOBILKOVA<sup>1</sup>, J. EHRMANN<sup>1</sup>, P. KNIZETOVA<sup>1</sup>, V. KREJCI<sup>1</sup>, O. KALITA<sup>2</sup>, Z. KOLAR<sup>1</sup>

<sup>1</sup>Institute of Pathology and Laboratory of Molecular Pathology, Faculty of Medicine and Dentistry, Palacký University, Hnevotinska 3, Olomouc, CZ-775 15, Czech Republic, e-mail: ahlob@email.cz; <sup>2</sup>Department of Neurosurgery, Teaching Hospital, I. P. Pavlova 6, CZ-775 20, Olomouc, Czech Republic

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Astrocytomas, particularly high grade astrocytoma, are brain tumors with potent angiogenic activity. Our immunohistochemical study assessed vascular endothelial growth factor (VEGF), VEGF receptors (Flk-1, and Flt-1), the intermediate filamental protein nestin which plays a role in central nervous system development, and MMP-9, which belongs the family of matrix metalloproteinases implicated in tumor invasion and angiogenesis regulation. We investigated the expression of VEGF, its receptors, nestin and MMP-9 in astrocytomas and their correlation with tumor grade. We used paraffin-embedded samples from 66 patients, 29 with low grade (WHO-grade II) and 37 with high grade (WHO-grade III and IV) astrocytomas. Antibodies against VEGF, Flk-1, Flt-1, nestin, CD34 and MMP-9 were used, followed by standard indirect immunohistochemical methods. Expression of Flt-1 and Flk-1 showed no significant differences between low and high grade tumor groups. Expression of VEGF and MMP-9 was increased in the high grade group (p equal to or less than 0.026 and 0.024). Nestin expression in tumor astrocytes and endothelial cells increased in high grade group (p same 0.007 and 0.003). Higher expression of VEGF in high grade astrocytomas may subsequently lead to activation of survival, angiogenesis and migration. Expression of nestin and MMP-9 also suggest their likely role in astrocytoma vascular development and proliferation.

*Key words: astrocytoma, angiogenesis, VEGF, nestin, MMP-9 and immunohistochemistry.*

Astrocytomas, particularly high grade types are characterised by aggressive growth, tumor angiogenesis and limited response to radio and chemotherapy. The most frequent alterations in astrocytomas are described in cell cycle pathways (p16-cdk4-pRb and ARF-MDM2-p53) and growth factor-regulated signaling pathways [1, 2]. Angiogenesis is part of the picture in a number of pathological states, including chronic inflammatory diseases and tumor growth [3]. An association between microvascular density, tumor growth, metastasis and prognosis has been reported in several human tumors, including breast [4], stomach cancers [5], melanoma [6] and astroglial brain tumors [7]. Angiogenesis is a complex multistep process involving extracellular matrix remodeling, endothelial cell migration and proliferation, capillary differentiation and anastomosis. These processes are controlled by angiogenic factors produced by tumor cells [3]. Although the factors causing tumor angiogenesis are not completely understood, the current leading candidates include vascular endothelial growth factor

(VEGF), also known as the vascular permeability factor. VEGF is a multifunctional cytokine, endothelial cell-specific mitogen and angiogenesis inducer in vivo [8]. VEGF also increases vascular permeability and peritumoral oedema. The importance of VEGF as a mediator of tumor angiogenesis has been demonstrated in several investigations [9 – 13]. VEGF binds to two different high-affinity tyrosine kinase receptors: Flt-1 (VEGFR-1) and Flk-1 (KDR, VEGFR-2) expressed principally in vascular endothelium [14, 15]. Recently, neuropilin-1 has been identified as a third VEGF receptor, without tyrosine kinase function, and acts as a co-receptor enhancing binding to the Flk-1 [16].

Nestin is a protein belonging to class VI intermediate filaments and is produced by stem/progenitor cells in the mammalian central nervous system (CNS) during development and is replaced by vimentin and glial acidic fibrillary protein (GFAP) during neurocytogenesis [17]. In adult non-neoplastic brain tissue nestin is rarely detected, although it is sometimes found in endothelial cells [18]. It might be expected that CNS tumor cells would show a gene expression pattern similar to that of the cells of the developing CNS from which

\* Corresponding author

they arise. The matrix metalloproteinases (MMPs) are members of a family of at least 21 Zn<sup>2+</sup>-dependent endopeptidases, soluble or membrane bound [19]. The expression of most MMPs is regulated by a number of mechanisms: at mRNA level transcriptionally by cytokines, hormones, and growth factors. They play important roles in many normal biological and pathological processes, including wound healing, inflammation, angiogenesis, the invasive potential of many solid tumors and cancer metastasis [20]. The main characteristic of MMPs is degradation of the extracellular matrix of basement membranes which enables cancer cells to invade tissues. MMP-2 and MMP-9 members have been observed in glioma-derived cell lines [21]. Among MMPs, MMP-9 is particularly interesting because of a predominant localization in the region of angiogenesis [22]. There is some evidence that MMP-9 could be a potential angiogenic factor that signals through VEGF-VEGFR system [23]. CD34 antigen is a transmembrane protein, expressed on hematopoietic stem cells and lineage-specific progenitor cells, on a subset of bone marrow stromal cells and small vessel endothelium of a variety of tissues [24]. Detection of CD34 antigen has been used as a comparative marker to visualise the endothelial cells of newly formed blood vessels and is described in a number of publications [25 -27]. The aim of our study was to investigate the significance of VEGF expression, its receptors, nestin and MMP-9 proteins and differences in low and high grade astrocytomas.

## Materials and methods

**Tissue samples.** We investigated astroglial tumor samples from 66 patients, divided into 29 low grade astrocytomas (WHO-grade II) and 37 high grade (WHO grade III and IV).

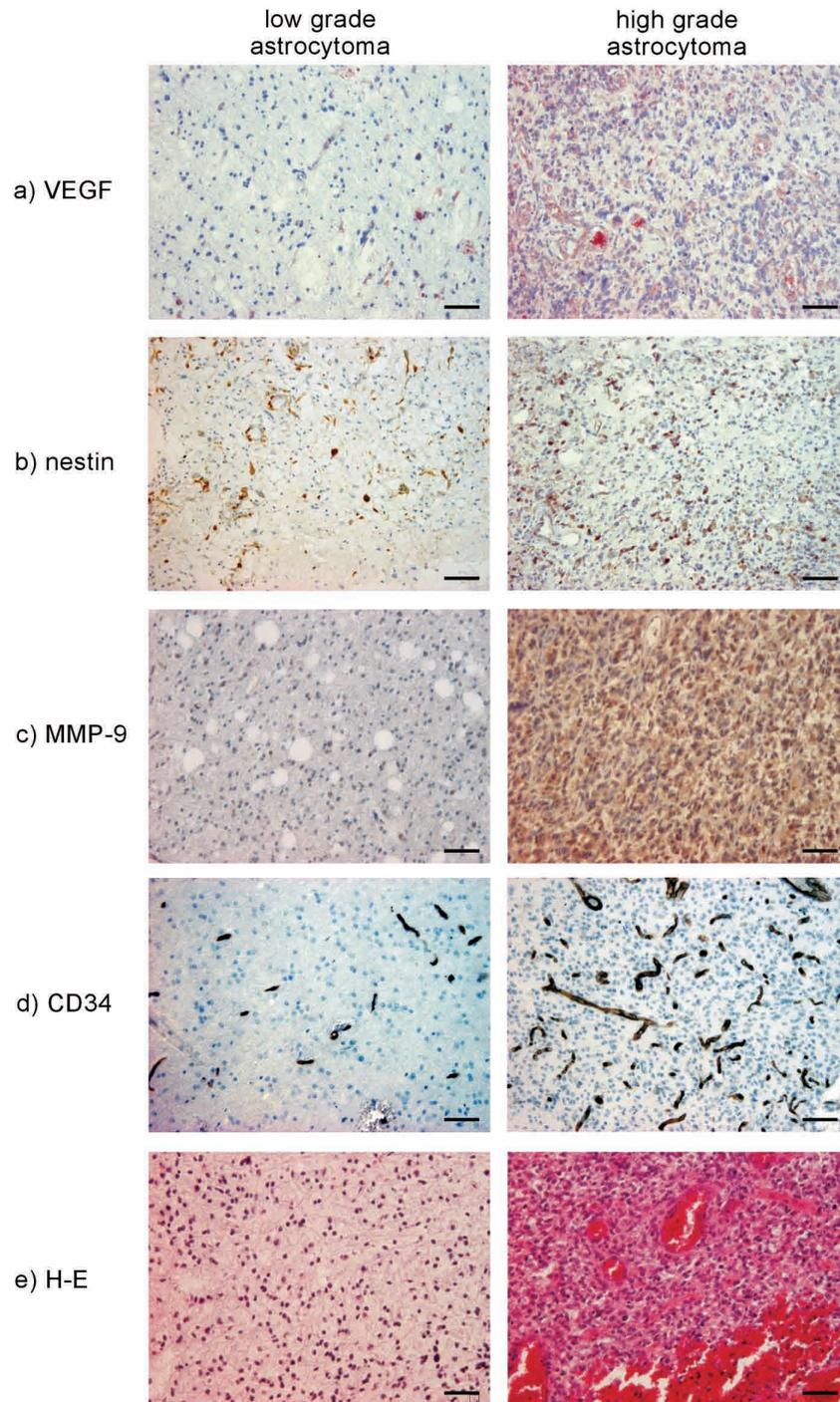
The material was routinely formalin-fixed and paraffin-embedded. Samples were taken from the archival collection of the Institute of Pathology of Palacký University over the period 1986-2004. Tumor samples were obtained from patients who had undergone surgery at the Department of Neurosurgery, Teaching Hospital in Olomouc, Czech Republic. Patients with astrocytomas WHO-grade II ranged from 11 to 80 years old, median (Md)=45,5 years and patients with astrocytomas (WHO grade III and IV) from 29 to 75 years, Md=52 years. In the group of low grade astrocytomas the ratio between the sexes was comparable (47% females and 53% males). 25 samples of astrocytomas WHO-grade II were diagnosed as fibrillary astrocytoma, 1 as gemistocytic astrocytoma, and 3 samples had a minor oligodendroglial component. In the group of astrocytomas WHO-grade III and IV males predominated with 73%. 8 samples were diagnosed as anaplastic astrocytoma (WHO-grade III), 26 as glioblastoma (WHO-grade IV) and 3 samples showed minor oligodendroglial component (1 grade III, and 2 grade IV). All cases were diagnosed according to the standard diagnostic criteria of WHO classification [28]. Tumors WHO-grade III and IV was evaluated in one category as "high grade" group, division on separated categories would lead to small number of patients particularly in category with WHO-grade III and would decreased statistical significance of the results.

**Immunohistochemistry.** One paraffin block having the most representative tumor area of each tumor was selected for immunohistochemistry. A summary of 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 methods with Envision plus kit labelled polymer

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

Antibody against to	Clone	Source	Concentration	Time of incubation	Antigen unmasking treatment
VEGF	mouse monoclonal, C-1	Santa Cruz Biotechnology, CA, USA	1: 25	60 min	1mM citrate buffer, pH 6, MW 20min, 110°C
Flk-1	mouse monoclonal, A-3	Santa Cruz Biotechnology, CA, USA	1: 50	60 min	1mM citrate buffer pH 6, MW 20min, 98 °C
Flt-1	rabbit polyclonal	NeoMarkers, CA, USA	1: 150	60 min	1mM citrate buffer pH 6, MW 20min, 98 °C
Nestin	mouse monoclonal	Chemicon, CA, USA	1: 100	60 min	1mM citrate buffer pH 6, MW 20min, 98 °C
CD34	mouse monoclonal QBEND10	Dako, Glostrup, DK	1: 50	60 min	1mM EDTA, pH 8, water bath, 40min
MMP-9	rabbit polyclonal	NeoMarkers, CA, USA	1: 50	60 min	1mM citrate buffer pH 6, MW 20min, 98 °C

MW – microwave generator, VEGF – vascular endothelial growth factor, Flk-1, Flt-1 – vascular endothelial growth factor receptors, MMP-9 – matrix metalloproteinase -9



**Figure 1.** Demonstration of immunohistochemical detection of the protein expression and hematoxylin-eosin (H-E) staining of low and high grade astrocytoma.

- VEGF detection shows increase expression in high grade astrocytoma. For detection was used mouse monoclonal antibody (C-1, Santa Cruz Biotechnology). Final magnification was 200 $\times$ , scale bar=50  $\mu$ m.
- Nestin detection shows high expression in high grade astrocytoma (in tumour astroglial cells and endothelial cells). For detection was used mouse monoclonal antibody (Chemicon). Final magnification was 200 $\times$ , scale bar 50  $\mu$ m.
- MMP-9 detection shows high expression in high grade astrocytoma. For detection was used rabbit polyclonal antibody (NeoMarkers). Final magnification was 250 $\times$ , scale bar=62,5  $\mu$ m.
- CD34 detection demonstrates increased microvascular density in high grade astrocytoma. For detection was used mouse monoclonal antibody (QBEND10, Dako). Final magnification was 200 $\times$ , scale bar=50  $\mu$ m.
- Hematoxylin-eosin staining, final magnification was 250 $\times$ , scale bar=62,5  $\mu$ m.

HRP (Dako, Glostrup, DK). Diaminobenzidine (Fluka, Buchs, Schweiz) or 3-amino-9-ethylcarbazole (Dako, Glostrup, DK) was used as chromogenic substrate and tissues were counterstained with hematoxylin (Merk, Darmstadt, Germany). For negative control, primary antibody was omitted. The slides were analyzed by two experienced pathologists (Tab. 1).

Immunohistochemical staining was evaluated by a semi-quantitative method using a histoscore (HS) which is a multiplication of positivity by intensity of staining [29]. Positivity of staining was assessed as percentage of positive cells. Intensity of staining was scored as weak – 1, moderate – 2 or strong – 3. Expression level of analysed proteins in some cases varied inside of one slide, HS show average value. Protein expression of VEGF, Flk-1, Flt-1 and nestin was analysed in the cytoplasm of astroglial tumor cells and endothelial cells separately. Protein expression of MMP-9 was analysed in the cytoplasm of astroglial tumour cells only. CD34 detection was used as a marker of endothelial cell.

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

**Statistical analysis.** t-test and non-parametric Mann-Whitney test were used to evaluate the data for low and high grade astroglial tumors and related variables. SPSS 10.1 was the software used and the level of significance was 0,05.

## Results

VEGF was detected in the cytoplasm of tumor astrocytes and in endothelial cells in a small part of the blood vessels. The median VEGF histoscores for astrocytes in the low grade group was 30.0, in the high grade group was 80.0. The difference was significant ( $p=0.026$ ) with using of non-parametric Mann-Whitney test. Figure 1a shows immunohistochemical detection of VEGF in both groups of astrocytomas. No differences were found in VEGF expression in endothelial cells between the two groups of astrocytomas. No statistically significant correlations were found between VEGF and nestin, VEGF and MMP-9 expressions in tumor astrocytes.

Flt-1 and Flk-1 were localised in the cytoplasm of neoplastic astrocytes. VEGF receptors were also detected in a portion of the endothelial cells in most analysed cases, with no significant differences in quantity of stained blood vessels or staining intensity. The median Flt-1 histoscore in tumor astrocytes for the low grade group was 20.0 and for high grade group 30.0. The difference was not statistically significant ( $p=0.384$ ) with using of non-parametric Mann-Whitney test. The level of Flk-1 expression was in general slightly higher than the level of Flt-1. Flk-1 histoscore in tumour astrocytes for the low grade group was 70.0 and for the high grade group was 80.0. The difference was not significant ( $p=0.859$ ) with using of t-test. Further, no correlation was found between VEGF receptors, VEGF, nestin and MMP-9 expression.

Nestin expression was detected in high intensity in the cytoplasm of neoplastic astrocytes and endothelial cells. The

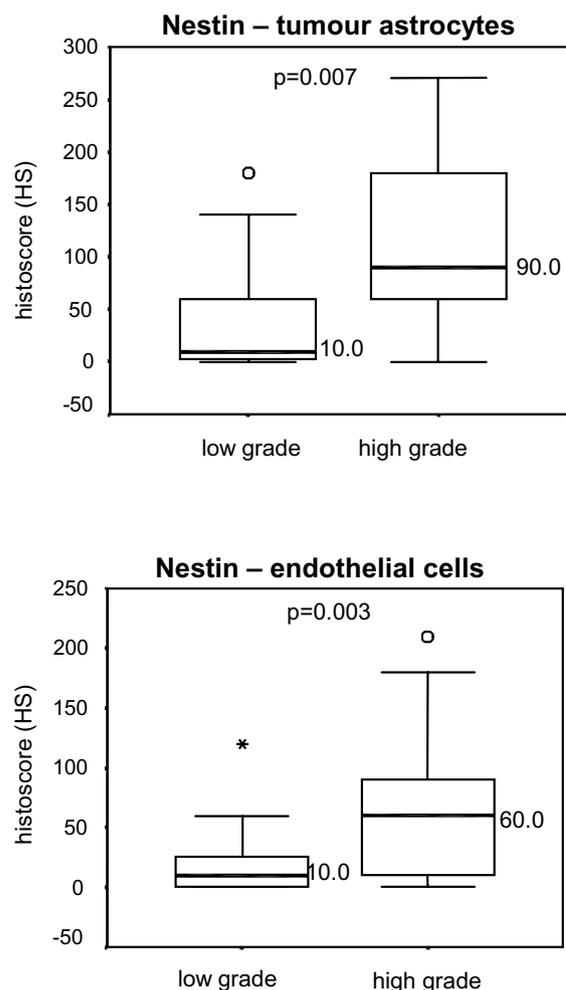
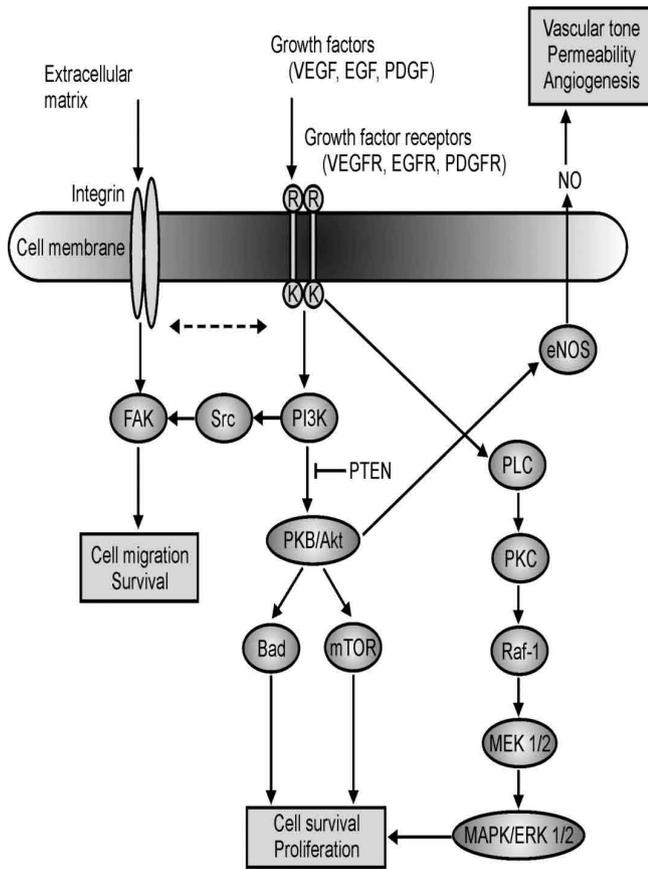


Figure 2.

- The graph shows the protein expression of nestin as a histoscore (HS) with minimal and maximal unoutlying values in astroglial tumor cells, in the group with low and high grade stage.
- The graph demonstrates the protein expression of nestin as a histoscore (HS) with minimal and maximal unoutlying values in endothelial cells, in the group with low and high grade stage.

The thick line inside of the box corresponds to median, bottom of the box to 1. quartile, head of the box to 3. quartile and altitude of the box to interquartile span – characterising variability of data. The circle represents outliers, asterisk extreme value.

median nestin histoscore in tumor astrocytes was 10.0 for the low grade group and 90.0 for the high grade group. The median nestin histoscore in endothelial cells was 10.0 for the low grade group and 60.0 for the high grade group. We found a positive relationship of nestin with tumor grade for nestin expression in both tumor astrocytes and endothelial cells ( $p=0.007$ , and  $p=0.003$ ) with using of non-parametric Mann-Whitney test. Figure 1b shows immunohistochemical detection of nestin in both groups of astrocytomas. Figure 2 shows box graphs with the median nestin as histoscore in tumor astrocytes 2a, and endothelial cells 2b.



**Figure 3.** The tyrosine kinase activity of growth factor receptor is stimulated upon binding of growth factors, which results in stimulation of multiple downstream signaling cascades. These pathways are important for a wide range of cellular functions, including cell survival, proliferation, angiogenesis and migration. Abbreviations: VEGF – vascular endothelial growth factor, VEGFR – vascular endothelial growth factor receptor, EGF – epidermal growth factor, EGFR – epidermal growth factor receptor, PDGF – platelet-derived growth factor, PDGFR – platelet-derived growth factor receptor, R – receptor, K – kinase, PI3K – phosphatidylinositol-3-kinase, PTEN – phosphatase and tensin homolog deleted on chromosome 10, PKB/Akt – protein kinase B, mTOR – mammalian target of rapamycin, eNOS – endothelial nitric oxide-synthase, NO – nitric oxide, PLC – phospholipase C, PKC – protein kinase C, MAPK – mitogen-activated kinase, ERK – extracellular signal-regulated kinase, MEK – MAPK/ERK kinase, FAK – focal adhesion kinase. Adapted from [38, 39 and Cell Signaling].

MMP-9 protein was found in the cytoplasm of neoplastic astrocytes and in most of the samples also in extracellular matrix. Staining of extracellular matrix was difficult to evaluate, both quantitatively and also in terms of intensity. For this reason, only cytoplasmic expression was followed. The median MMP-9 histoscore in tumor astrocytes was 100.0 for the low grade group and 140.0 for the high grade group. The protein expression of MMP-9 clearly increases with grade of tumor ( $p=0.024$ ) with using of t-test. Positive correlation between MMP-9 and nestin ( $p=0.07$ ) shows to be near to significant

level. Figure 1c shows immunohistochemical detection of MMP-9 in both groups of astrocytomas.

## Discussion

The VEGF gene is normally expressed in non-malignant adult human tissues including lung, kidney, adrenal gland, liver, heart and stomach. The prominence of VEGF mRNA in these tissues suggests that VEGF plays a role in regulating the baseline microvascular permeability of the normal microcirculation, which is essential for tissue nutrition and waste removal [30]. VEGF mRNA level is substantially elevated in several tumors, fibrosarcoma, osteosarcoma, colonic adenocarcinoma, clear cell type of renal carcinoma, compared with normal tissue [30]. In this study we found protein expression of VEGF in cytoplasm of astrocytes and endothelia in most analysed astroglial brain tumors. Cytoplasmic expression of VEGF and its receptors was also observed by Knizetova et al [31] in astroglial cell lines and tissues from astroglial tumors. VEGF expression showed significant increase in the high grade stage. Similar result demonstrates reports on increased VEGF expression in association with a higher risk for relapse or as a negative prognostic factor in non-small cell lung cancer [32], estrogen receptor-positive breast carcinoma [33] and gastric cancer [34]. Huang et al [35] found that of several types of primary and secondary brain tumors the highest expression of VEGF in high grade astrocytomas. VEGF receptor expression in the group of astroglial tumours correlated with tumor malignancy [35]. We predicted that the expression of VEGF receptors would positively correlate with tumor grade in our study. The results, however, show almost the same histoscore for Flt-1 and Flk-1 for both tumor grades. We found no correlation between expression of VEGF receptors and VEGF. We do not have clear explanation for such different results on VEGF receptors protein levels obtained by Huang et al [35] and our working group. We can think about different approaches to protein detection where the ELISA method was used in Huang's group and this may be more sensitive and detect protein below the threshold for immunohistochemistry and/or using different clones of primary antibodies. Decaussin et al [36] described a positive correlation for VEGF and Flt-1 protein expression, but not for VEGF and Flk-1, in a samples of non-small cell lung carcinomas. Data on the correlation of VEGF and VEGF receptors from different tumors are variable. Although we found no correlation between VEGF and VEGF receptor expression, generally, in both, low and high grade tumors, at least part of astrocytes and endothelial cells co-expresses VEGF and its receptors. We can conclude that a loop, may exist where astrocytes utilize VEGF for autocrine growth and the neoangiogenesis could be partly regulated in a paracrine manner, where VEGF may be produced in tumor astrocytes and then be bound to the endothelial cells to allow their growth via induced VEGF receptors. However, there probably exists another mechanism as well, which promotes tumor astrocyte proliferation and neoangiogenesis. Treatments

which retard tumour angiogenesis targeted on VEGF using anti-VEGF antibody (as e.g. bevacizumab) [37-39], could benefit particularly patients with high grade astrocytomas.

SCHIFFER et al [40] described relatively variable expression of nestin in a sample of 50 gliomas with heterogeneous pattern of intensity in glioblastomas and also they describe nestin expression in proliferating endothelial cells. The astrocytic-restricted nestin expression was confirmed by negative staining of normal and neoplastic oligodendrocytes [40]. Mokry et al [41] reported expression of nestin in immature endothelial cells of embryonic capillaries generated in the course of angiogenesis, and only sporadic expression in mature adult human endothelial cells. They also demonstrated nestin positive endothelial cells which nourish solid growing tumors, including melanoblastoma and glioblastomas. Their data provide evidence that nestin participates in the formation of the cytoskeleton of newly formed endothelial cells under normal physiological and pathological conditions of vascular development. Our results supplement published data, and show a strong positive relation between nestin expressed in tumor astrocytes and endothelial cells, and tumour grade. Nestin positivity in tumor cells could emphasize their origin in multipotent precursor cells, and their astrocytic nature also. Precursor cells contribute to the formation of radial glia, which might represent the possible origin of gliomas [42]. Nestin could also represent a novel marker of neovascularisation [41, 18]. Pagestencher et al [21] demonstrated distinct expression of MMPs and their tissue inhibitors in a group of brain tumors, including astrocytomas, oligoastrocytomas and ependymomas. Immunohistochemical detection showed that MMP-9 was localised to vessel walls, to neutrophils in areas of haemorrhage, and in glioblastomas [21]. Forsyth et al [22] analysed cDNA from 46 brain gliomas and showed significantly elevated levels of MMP-2 in low and high grade gliomas, in comparison with normal tissue. MMP-9 was also observed higher in gliomas than in normal brain tissue. MMP-9 was expressed in tumor cells and blood vessels and more strongly correlated with tumor grade [22]. We demonstrated on our astrocytoma collection significant positive correlation of MMP-9 expression and tumor grade which is in agreement with Pagestencher and Forsyth et al [21, 22]. These data suggest that MMP-9 plays an important role in the pathophysiology of human gliomas and could be involved in both tumor invasion and angiogenesis.

To recapitulate, VEGF expression increases with astrocytoma grading and may subsequently lead to gene expression, DNA synthesis, activation of survival and migration via the protein kinase B (PKB/Akt), the mitogen activated kinase (MAPK) and focal adhesion kinase (FAK) pathways (Fig. 3). Results on nestin expression support a hypothesis about the origin, mainly of high grade astrocytomas, in multipotent neural cells and nestin is also a reliable marker of neovascularisation in astroglial tumors. We demonstrated that VEGF, nestin and MMP-9 take part in positive regulation of neoangiogenesis and proliferation during astrocytomagenesis.

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