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Prognostic Significance of Immunohistochemical Markers in Glioma Patients

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1. Introduction

Gliomas are the most common form of brain tumors, contributing to more than half of the incidence of brain tumors. They are derived from three basic types of glial cells: astrocytes, oligodendrocytes and ependymal cells. The most frequent are diffusely infiltrating astrocytomas, further classified into astroytomas (A), anaplastic astrocytomas (AA) and glioblastoma, equivalent to World Health Organization (WHO) grade II, II and IV, respectively (Kleihues & Cavenee, 2000). The term glioblastoma is used synonymously with glioblastoma multiforme (GBM), which suggests, the histopathology of this tumor is extremely variable (Kleihues & Cavenee, 2000). GBM is the most common and lethal type of astrocyte-derived tumor, corresponding to 50% of adult primary brain tumor cases, followed by anaplastic astrocytoma (30%) and astrocytoma (20%) (Greenberg, 2010). GBM may develop from astrocytoma or anaplastic astrocytoma (secondary GBM), but more frequently they manifest after a short clinical history *de novo*, without any evidence of a less malignant precursor lesion (primary GBM) (Farhadi & Rutka, 2008). Although primary brain tumors are relatively rare compared with carcinomas, they are characterized by higher mortality rates and increased disability. The overall annual incidence rate of primary malignant and benign brain tumors in developed countries is approximately 15 per 100,000 individuals, and for primary malignant brain tumors it is 7 per 100,000 (Minn et al., 2008). Brain tumor incidence and mortality have increased by up to 300% over the past 3 decades primarily in people aged over 75 years (Davis et al., 1996 as cited in Minn et al., 2008; Wrensch et al, 1993 as cited in Minn et al., 2008).

Malignant gliomas are among the most challenging of all cancers to treat successfully. The tumor cells vigorously invade surrounding tissue, which renders complete surgical resection difficult and contributes to the high incidence of the recurrence (Merzak et al., 1995). Invasion of glioma cells into adjacent brain tissue is dependent on their interaction with the extracellular matrix (ECM) and possible destruction of matrix barriers (Pilkington, 1994). Tumor cells at the invasive front have to detach from the primary tumor mass and re-attach to ECM components or to surrounding tissue elements. In general, invasiveness may result in deformation and destruction of the brain architecture which leads to the fatal outcome for the patient. Proteolytic modification of ECM components, such as laminin and

fibronection, is believed to facilitate the invasive spread of tumor cells (Gladson, 1999; Goldbrunner et al., 1999). Lysosomal cysteine cathepsins have been implicated in tumor progression. Proteolytic enzymes, including cahtepsins (Cats), mediate the invasion process, either acting alone or participating in proteolytic cascades (Schmitt et al., 1992; Sloane et al., 1994). Increased activity of proteolytic enzymes is observed during brain tumor progression (Frosch & Sloane, 1998; Levičar et al., 2003). Cathepsins are responsible for intracellular protein turnover and are vitally important for normal cell and organ development. The activity of the cysteine cathepsins can be regulated at various levels, ultimately by their endogenous inhibitors (Lah & Kos, 1998; Calkins & Sloane, 1995). In brain tumors, down-regulation of the total inhibitory activity of cystatins has been observed, presumably contributing to tumor malignancy (Sivaparvathi et al., 1996a).

In addition, tumor growth is critically dependent on blood supply and the development of new capillaries. In the case of tumor cell-induced angiogenesis, endothelial cells invade surrounding tissue in a process similar to that observed for tumor cells (Paku, 1998).

Human GBM also contains a various amounts of brain tumor stem-like precursor cells (BTSC) (Singh et al., 2003), which indicates a hierarchical model of tumorigenesis. The BTSCs display self-renewal potential, ex vivo multipotency and, most importantly, the ability to establish and expand the tumor in vivo. Moreover, genetic analyses of the BTSC in various patients have revealed distinct patterns of up-regulated genes, including patient-specific genes expression (Galli et al., 2004).

Despite recent advances in neuro-imaging, neurosurgical resection techniques and the development of novel adjuvant therapies, the long-term survival of patients suffering from malignant glioma remains low. Although treatment with temozolomide and radiotherapy improved median survival after diagnosis of GBM from 12 months to 14 months (Kalkanis & Rosenblum, 2008), the survival rate still ranges from a few months to several years, which, together with the poor prognosis, points the need for new, independent prognostic factors that may enable individualized treatment modalities, including molecular based therapies, of patients with unfavourable prognosis. Our studies are aimed to reveal differential expression and compare the prognostic significance of potential biological markers in glioma patients.

2. Immunohistochemical investigation of human gliomas

High-grade gliomas often demonstrate immunoreactivity for various markers. Such biologic markers could be used to assess the patient prognosis and may thus provide the new information regarding the time of glioma recurrence. In recent years we investigated the possible prognostic significance of different biological markers. The particularity of present report is the fusion of author's clinical work with his own experimental studies on animal models.

2.1 The animal models for experimental studies of human malignant brain tumors

The objective of experimental neuro-oncology is to contribute to a better understanding of human malignant brain tumors (Wechsler et al., 1989). To this end, the development of several animal models has provided specific clues about the formation of gliomas (Pilkington et al., 1997). Such animal models are also beneficial for selective molecular and biochemical analyses of tumor markers (Brem & Sawaya, 2004).

Previously, either commercial cell lines derived from human GBM or multicellular tumor spheroids from human gliomas have been used as a model for the study of brain tumor invasion (Bjerkvig et al., 1990; Engebraaten et al., 1999). Since all these cell types can grow in vitro, the cultures must be characterized to ascertain the cell subpopulations which have been selected through the culture conditions. Morphological characterization is not sufficient and a panel of markers may be required to define the populations present. However, as the cells adapt to the tissue culture conditions, they may lose the ability to express one or more of these markers. The need for better and more relevant brain tumor models is generally acknowledged.

2.1.1 U87 human glioblastoma cell xenografts in rat brains and on the chicken chorioallantoic membrane

The objective of our experimental work was to develop a simple and cheap animal model, with high tumor take rate, for brain tumor progression studies (Strojnik et al., 2006; Strojnik et al., 2010). A tumorigenesis model was presented, originating from tumor spheroids prepared from U87 human glioblastoma cell line, in the brain of rats and on the chick chorioallantoic membrane (CAM). U87 cells are considered to be a rapidly proliferating cell line, which can be grown in culture as monolayers and tumor spheroids. The U87 cell suspension, or precultured U87 tumor spheroids, was inoculated into the brain of 4-week-old rats and on CAM on embryonic day seven.

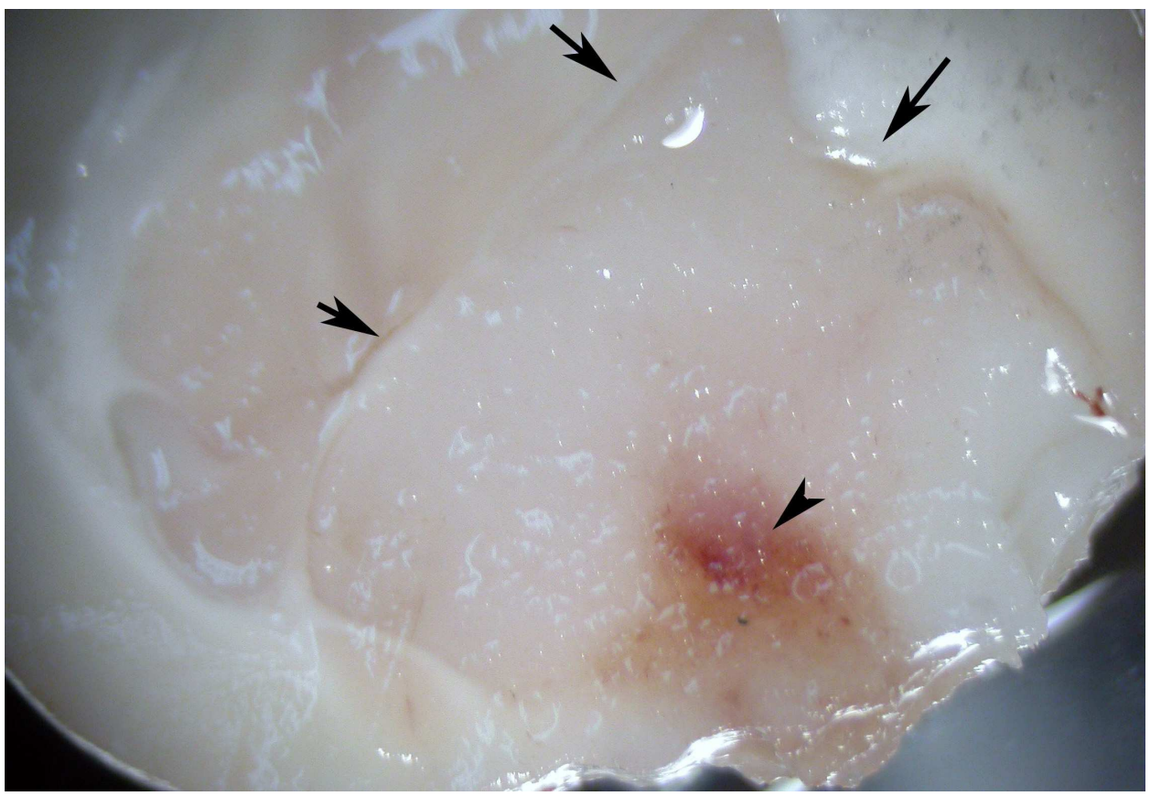


Fig. 1. Coronal rat brain tissue sections demonstrated solid, well demarcated, grayish tumor with an area of hemorrhage (arrow).

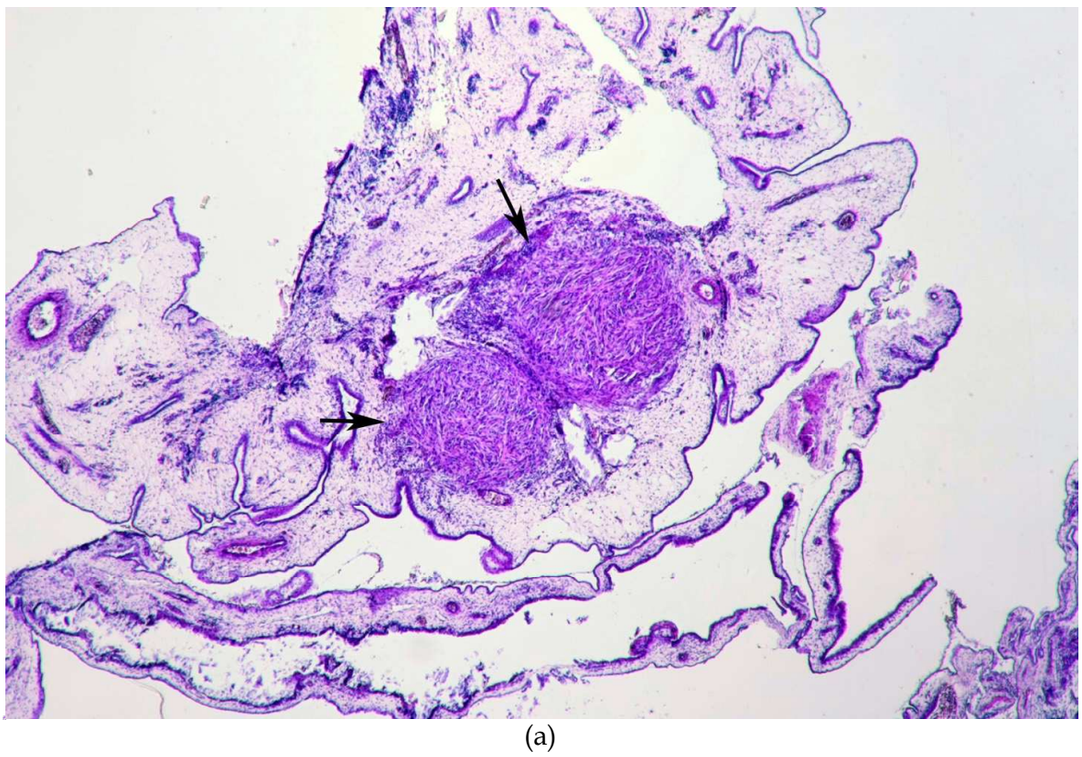
The resulting first generation tumors that were induced in rats were also transferred through serial transplantations from rat to rat, to obtain second and third generation tumors. The macroscopic tumor appearance, histopathology and immunohistochemistry of selected relevant tumor progression markers were monitored in U87 human glioblastoma cells xenografted into the brain of rats. In accordance with other reports, maximum progressive tumor growth was observed after 2 - 3 weeks (Wechsler et al., 1989). According to the WHO classification of human brain tumors (Kleihues & Cavenee, 2000), the transplantation tumors demonstrated features of anaplastic astrocytic tumor (WHO grade III), but become increasingly similar to glioblastomas (WHO grade IV) in the second and third generations. Parallel to this progression, increasing neovascularization and tumor necrosis was observed, both characteristic of glioblastoma. The benefits of inoculation of pre-cultured small tumor spheroids have been well described (Bjerkvig et al., 1990; Engebraaten et al., 1999). However, it should be stressed that spheroids from a biopsy specimen consist of heterogeneous cell populations, e.g. vascular elements (endothelial cells, pericytes), other mesenchyme-derived cells (fibroblasts) and microglia (a type of differentiated tissue macrophage) (Bressler et al, 1985; Badie & Schartner, 2001), requiring more careful characterization of the cellular composition of the inoculated spheroids when interpreting the resulting tumor behavior. In contrast, U87 cell-inoculated tumors have a relatively isomorphos cellular composition, due to their clonal origin, and further tumor development can be followed during subsequent generations, with respect to dedifferentiation of the tumor cells and induced recruitment of stromal cells from the tumor microenvironment (Mueller & Fusenig, 2004). The panel of marker protein expressions were followed in the first, second and third generations of tumors.

Anyway, the rodent models are somewhat limited by costs, experimental duration, variability and major ethical concerns, as well as by the difficulty of obtaining morphological data during tumor progression, resulting in large numbers of animals required to obtain conclusive results (Hagedorn et al., 2000). Another system consists of a human tumor grown in a xenogeneic host, the chick embryo. Consequently, we developed a tumorigenesis model, originating from the U87 human glioblastoma cell line, on the CAM, a densely vascularised extra-embryonic tissue (Strojnik et al., 2010). A few studies in chick embryos have been undertaken by others, but the primary focus was to demonstrate metastatic potential and not local invasiveness (Chambers et al., 1990; Ossowski & Reich, 1980). Our study aimed to compare the expression of various immunohistochemical markers of U87 cells and spheroids in culture and in rat brain with those grown on the CAM membrane. The macroscopic tumor appearance, histopathology and immunohistochemistry of selected relevant tumor progression markers were monitored in U87 human glioblastoma cells xenografted on the chick embryo CAM. In accordance with other reports, tumor growth was observed and well established tumors were seen after seven days in the CAM model.

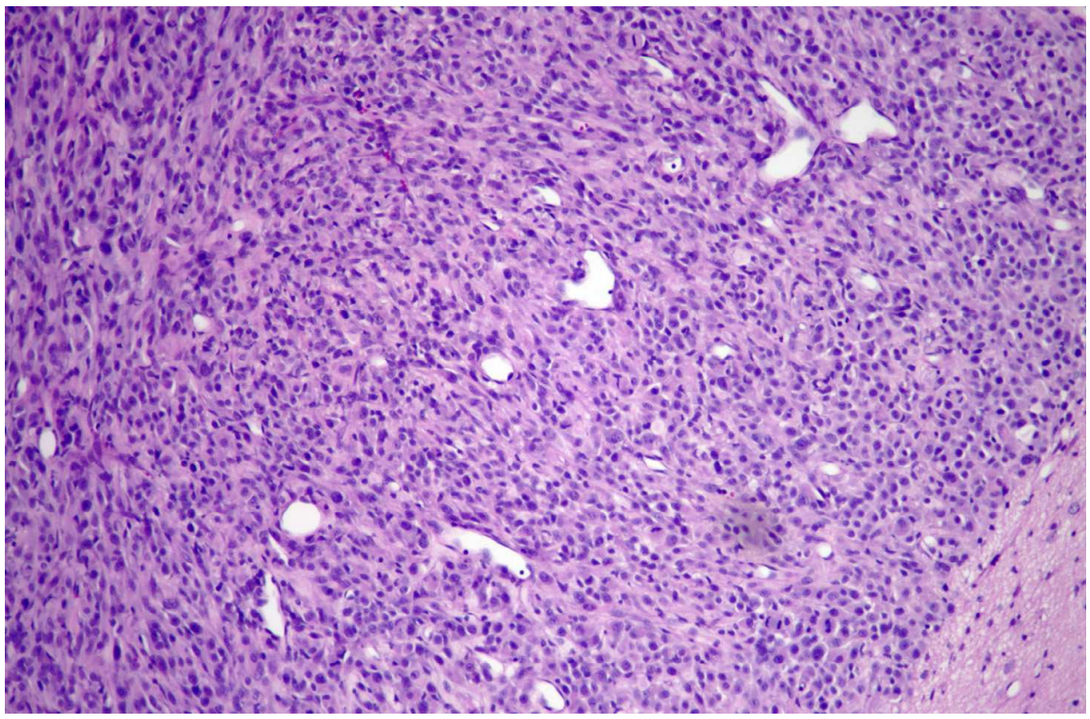
There were few differences in histological appearance between tumor models. In both tumor models, the full range of cytological features of human glioblastomas were observed, including astrocytes, small anaplastic cells, spindle cells and giant cells. In rats, tumors were more sharply demarcated from the surrounding brain, while on CAM, tumor nodules grew, with smaller groups of tumor cells growing apart from the gross tumor mass. These cells were usually within the connective tissue with some also attached to the vessel walls. This might be the result of how the tumor cells initially seeded upon inoculation; however, it may possibly mean that this model is more suitable for studying tumor invasiveness.



Fig. 2. Tumor nodules were seen on CAM one week after inoculation of U87 cells. Note small satellite tumors apart from the main tumor mass



(a)



(b)

Fig. 3. Within the CAMs, loosely connective tissue with blood vessels two tumor nodules can be seen (arrows) (H&E, x4) (a); all tumors in rats were sharply demarcated against the surrounding brain tissue (H&E, x40) (b)

The immunohistochemical staining of animal samples was performed for Ki-67, p53, vimentin, glial fibrillary acidic protein (GFAP), S100, CD3, CD20, synaptophysin, cathepsin B, cathepsin L, CD68, vascular endothelial growth factor (VEGF), and leukocyte esterase. The staining of rat brain sections for nestin, musashi and kallikrein 6 was also done.

Ki-67 antigen expression is a measure of the proportion of cellular and, hence, biological aggressiveness in malignancy (Scott et al., 1991; Wilson et al., 1996). The index of proliferation, Ki-67 LI, was high in all samples, but the fraction of Ki-67-positive cells was higher in U87 cell suspension and in rats compared to the spheroids and CAM tumors, indicating increased proliferation in the rat model of tumorigenicity.

The *p53* tumor suppressor gene is frequently mutated in glioblastomas (Newcomb et al., 1993). Mutations within the *p53* gene often results in aberrant expression of the p53 protein, leading to protein accumulation within the nucleus of the cells. The p53 protein is involved in regulation of the cell cycle and it has been speculated that the presence of abnormal amounts of p53 protein is associated with increased rates of proliferation (Cunningham et al., 1997). It was found that the percentage of p53-positive nuclei, which was higher in the tumors grown on the CAM than on rats, did not correlate with the Ki-67 LI. Other researchers have also reported little correlation between this pair of immunohistochemical markers (Cunningham et al., 1997; Balčiūnienė et al., 2009).

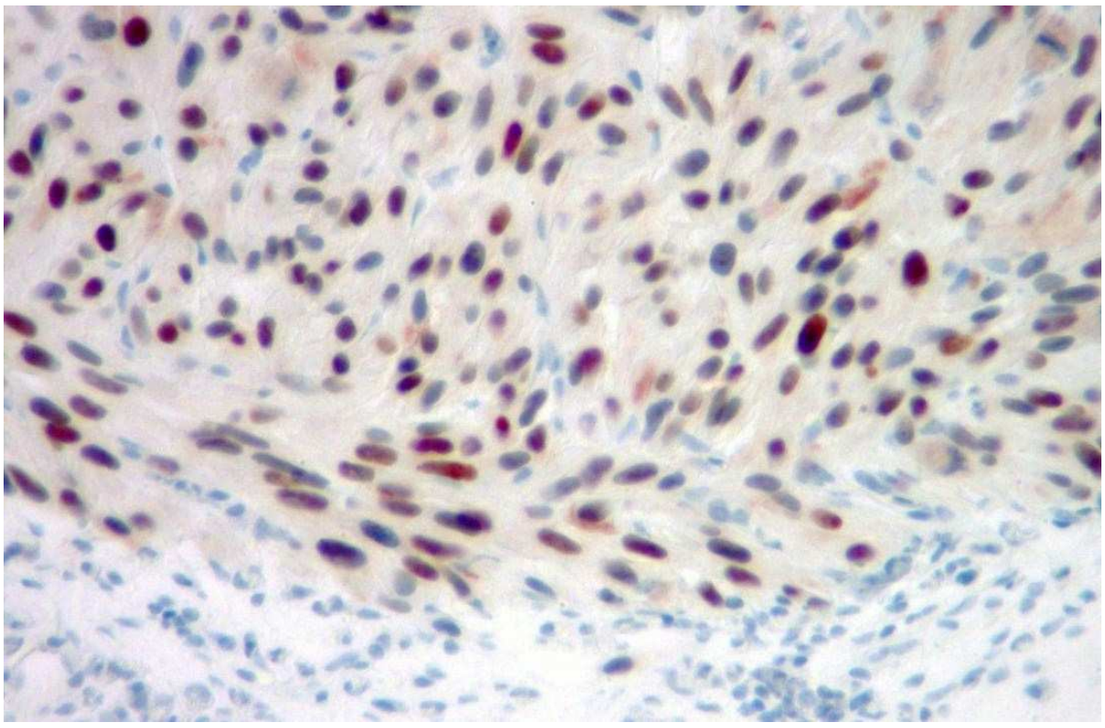


Fig. 4. In tumor grown on CAM many nuclei stained positively for p53 (x40)

Vimentin is an intermediate filament protein, which marks the mesenchymal cell phenotype. In the course of development of the nervous system, vimentin appears first in immature glial cells (Dahl et al., 1981), but rapidly decreases as glial fibrillary acidic protein (GFAP) appears concomitantly with myelination (Dahl, 1981). In mature astroglia, vimentin and GFAP coexist, and normal, reactive and neoplastic astrocytes have been found to contain variable amounts of both (Yung et al., 1985). In our works (Strojnik et al., 2006; Strojnik et al., 2010) it was revealed that high production of vimentin by the tumor cells was preserved in the host in both animal models, thus showing that the U87 clone consists of immature cells. Due to strong and specific staining of the tumor cells, it facilitated visualization of satellite tumors and migrating tumor cells away from the main tumor mass.

GFAP is mostly restricted to mature astrocytes (Lazarides, 1982). In human malignant gliomas, co-expression of GFAP and vimentin has been reported (Herpes, 1996). This was not the case in the animal models used in our study (Strojnik et al., 2010). Although the U87 cell suspension presented a moderate immune reaction for GFAP, this was completely absent from the induced tumors. This does not necessarily mean that a cell is of non-glial origin, but the ability to synthesize GFAP after further dedifferentiation of the U87 cells in the host is gradually lost.

The S100 family of calcium-binding proteins contains approximately 16 members, each of which exhibits a unique pattern of tissue/cell type-specific expression. Although the distribution of these proteins is not restricted to the nervous system, the implication of several members of this family in nervous system development, function, and disease has sparked new interest in these proteins. Different forms of malignant tumors exhibit dramatic changes in the expression of S100 proteins (Sedeghat & Notopoulos, 2008; Sen & Belli, 2007). Only moderate S100 immunoreactivity was detected in the U87 cell suspension and weak

expression in the spheroids. Tumors in both animal models were S100 negative, which can be explained by the possible down-regulation of S100 expression following tumor dedifferentiation (Bressler et al., 1985).

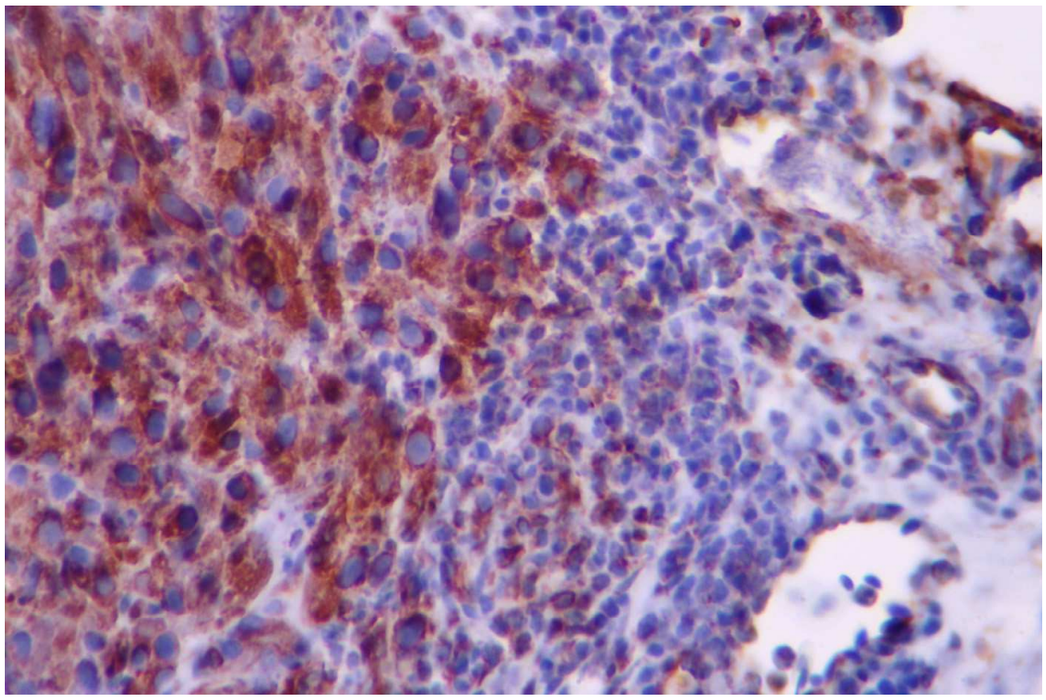


Fig. 5. All of the CAM tumor cells presented a strong and specific immune reaction for vimentin. Vessel walls stained positively for vimentin also (x40)

CD3 is a marker of T lymphocytes. Heterologous transplantation of human tumor cells into animals inevitably leads to immunological response of the host. Cytotoxic T lymphocytes have been implicated as the effectors cell mediating graft rejection (Bierer et al., 1985). Only small numbers of CD3-positive cells were seen in tumor grown on CAM, whereas in rats more CD3-positive cells were seen indicating that some sort of immune response is also present in this immunoincompetent hosts or that these CD3-positive cells might be "T-like" cells, but not actual T-cells, which adopted T lymphocytes phenotype during the dedifferentiation. As expected, no CD3-positive cells were observed in suspension, neither in spheroids nor outside the tumor in the host.

CD20 is expressed on all stages of B cell development, except the first and the last stages. It is also found on skin melanoma cancer stem cells (Fang et al., 2005). No CD20-positive cells were detected in any of the models used in our study. This might be partially explained by the fact that neither B lymphocytes nor antibodies in the circulation nor in the graft itself are required for first-set graft rejection (Hall et al., 1978).

Synaptophysin is a reliable marker for the identification of normal neuroendocrine cells and neuroendocrine neoplasm (McKeever, 1998). Only weak staining was observed in some of the tumor cells in suspension and in spheroids, but no staining was observed in animal tumor models, indicating that tumors induced by U87 clone underwent further dedifferentiation in the host. In humans, pure glial tumors do not usually express synaptophysin.

As expected, our study found strong Cat B staining in culture and in all of the animal models, where not only tumor cells but also endothelial cells stained positively for Cat B. Interestingly, stronger staining for Cat B in tumors was noted in rats than in chicken embryos. This could partially be explained by the relative ease of spreading of the tumor cells in the loose connective tissue of the CAM compared to the brain where more proteolytic activity is needed. We found Cat L staining in tumor cells in culture and in both animal models. Stronger staining for Cat L in tumors was noted in chicken than in rats. Staining with Cat L antibody revealed strong reaction in tumor cells, but there was no staining in the vascular endothelia. Stronger Cat L staining has been noted in the tumor centre, indicating slightly different roles of two cysteine proteases (Cat B and Cat L) in local invasiveness and in the malignant transformation of brain tumor cells.

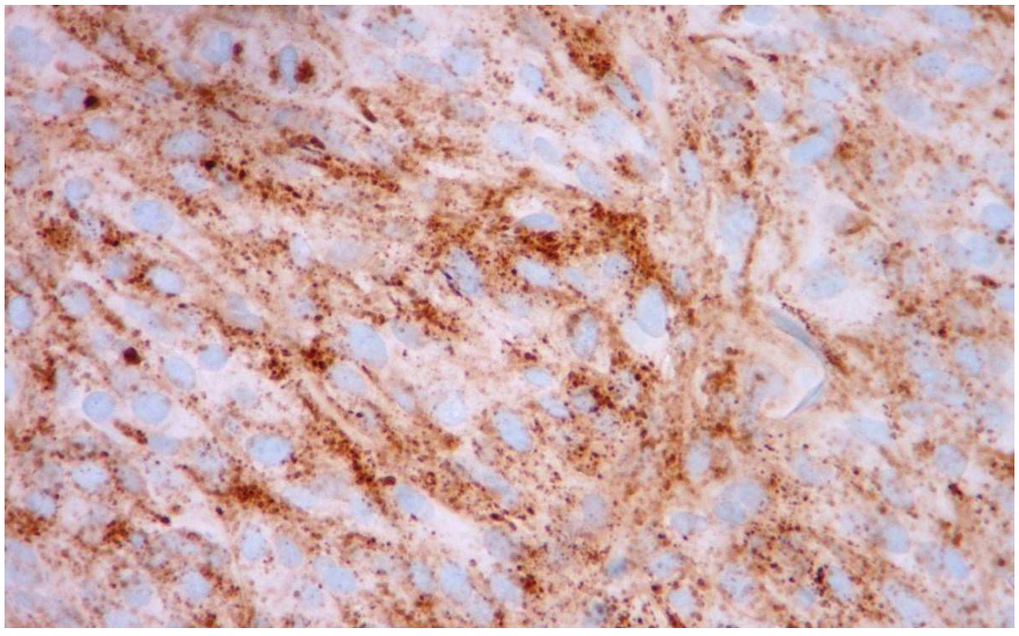


Fig. 6. Strong immunostaining for Cat B was noted in all samples, especially in rats. Vascular endothelia also stained positive for Cat B (40x)

CD68 is a specific marker for macrophage and also resting microglia (Hulette et al., 1992). Considered as immune effector cells of the CNS, the microglia represent a major component of the inflammatory cells found in malignant gliomas (Badie et al., 1999). In accordance to others (Leenstra et al., 1995) our studies also found strong CD68 expression in U87 human glioblastoma cell suspension, in U87 spheroids, as well as in rat and chick embryo U87 tumors (Strojnik et al., 2006; Strojnik et al., 2010). They stained for CD68 in the same way as macrophages do. Again, only minor differences in CD68 expression between animal models were noted. In accordance with the strong immune reaction for CD68 in all our samples we believe, that tumor cells adopted macrophage phenotype due to dedifferentiation.

Vascular endothelial growth factor (VEGF) is a potent mitogen specific for vascular endothelial cells and may directly stimulate the growth of new blood vessels (Leung et al., 1989). Angiogenesis is induced by tumor cell hypoxia and pro-angiogenetic factors (Hendriksen et al., 2009). Both brain tumor models showed only low VEGF expression. The slightly higher levels of vascular endothelial proliferation seen in rat tumors may be

explained by the likelihood of greater hypoxia associated with larger tumor size. It is well known that hypoxia is a potent VEGF trigger.

Leukocyte esterase is an enzyme present in most white blood cells. Neutrophils are present in glioblastoma tissue and not limited to necrotic areas. Researchers reported correlation between tumor grade and the extent of the neutrophil infiltration (Fossati et al., 1999). Their role in glioma progression remains unclear. Extensive leukocyte infiltration was observed in the brain tumor models in our study (Strojnik et al., 2010). This might partially be explained by the fact that xenotransplant acts as a foreign body in both animal models, and perhaps that esterase acts similarly to cathepsins thus contributing to the degradation of extracellular matrix.

Nestin has been detected in primary CNS tumors (Dahlstrand et al., 1992). We showed that nestin could be used as a biological marker for glioma malignancy (Strojnik et al., 2007). Contrary to the weak nestin expression of the U87 clone in cell cultures, immunostaining was absent in the spheroids. A switch to further dedifferentiation in the host might explain the moderate staining in the first generation tumors induced by inoculation of U87 cells or spheroids, as well as in the second and third generations of tumors induced by implantation of tumor tissue. The U87 cell suspension presented a weak immune reaction for musashi and, in contrast to nestin, its expression increased in the tumor cells as the tumor progressed, perhaps related to their malignant growth and dedifferentiation.

Tissue kallikreins have recently been strongly associated with tumor progression (Diamandis et al., 2004). Most of the tumor cells in suspension, spheroids and in tumors of all three generations presented a strong immune reaction for kallikrein 6, whereas the expression in the vascular endothelia of the tumor and in the surrounding brain was weak or absent. Normal and edematous rat brain tissue showed weak expression of kallikrein. Strong kallikrein 6 immunostaining in the U87 cell suspension and in tumors induced by injection of spheroids and implantation of tumor tissue indicated the role of kallikrein in tumor growth.

In conclusion, both animal models, the U-87 human glioblastoma spheroid cell line inoculated in rats or onto the chick embryo CAM, provide a good system for experimental studies of human malignant brain tumors. The panel of marker protein expression was followed in animal tumors. The data from rat study indicated that tumor progression was characterized by increased cell proliferation and tumor cell dedifferentiation, but lower invasiveness of the resulting tumors. Increased angiogenesis indicated high malignancy of the higher tumor generation. Data from the comparison of a panel of immunohistochemical markers between the CAM and rat models indicates that tumor protein expression in the CAM model is sufficiently similar to the rat model. We believe that the chick embryo CAM model is a good alternative to rodent brain tumor models. Anyway, both models may provide the basis for multigenetic and multimolecular glioma tumor cell analyses. They also have a potential use in testing individualized therapies.

2.2 Immunohistochemical staining and prognostic impact of biological markers in glioma

More than hundred patients with primary tumor of central nervous system (CNS), operated at our institution were studied. The histological slides of all cases were reviewed and classified according to the WHO classification of brain tumors (Kleihues & Cavenee, 2000). Patients' clinical and radiological data were collected, including age, sex, date and type of

initial operation, clinical neurological examination, computer tomography (CT) features and data of adjuvant therapy. For the survival analyses the follow-up data were registered. Immunohistochemical staining was performed using the standard technique (Strojnik et al, 1999; Strojnik et al., 2005). The staining for markers was scored separately for the tumor cells, the endothelial cells, and/or the macrophages, as described previously (Strojnik et al, 1999; Strojnik et al., 2005; Strojnik et al., 2007; Strojnik et al., 2009). IHC staining was performed for various marker including cathepsins B and L, nestin, musashi, CD68, kallikrein 6 and Ki-67. Statistical analysis was performed using the program Statistica for Windows 6 (StatSoft, Inc., Tulsa, OK, USA). Overall survival probabilities were calculated by the Kaplan-Meier Method (Kaplan & Meier, 1958, as cited in Strojnik et al., 1999); log-rank test was used to evaluate the association between survival and each of the selected markers.

It is unique that the panel of markers was tested on the same group of patients. The fact is that many published biomarker studies in gliomas investigate one, two or maybe three potential markers in their tumor samples. In our successive investigations of numerous markers we were working on the same group of glioma patients, what enables us to compare the prognostic significance of different tumor markers.

2.2.1 Cysteine proteinases cathepsins B and L

Endopeptidases, including cystein proteinase cathepsin (Cat) B, are suggested to be useful prognostic factors in many types of cancer (Lah & Kos, 1998). McCormick first found that Cat B is abundantly secreted in human gliomas in vitro as a latent zymogen requiring activation (McCormick, 1993). Cat B expression at the protein and mRNA levels was later shown to correlate with the malignant progression of gliomas (Rempel et al., 1994; Sivaparvathi et al., 1995). The first immunohistochemical (IHC) study on Cat B in gliomas was performed by Mikkelsen et al. (Mikkelsen et al., 1995), which not only confirmed such an association, but also found high Cat B expression in endothelial cells of new vasculature within the tumors.

In our study we have demonstrated that cathepsin B is expressed in glial tumor cells, macrophages near vessels adjacent to necrotic area, and proliferative endothelial cells of primary tumors of the CNS (Strojnik et al., 1999). Significantly more cases with high Cat B IHC score in tumor and in endothelial cells were observed in malignant compared with benign tumors.

Our results confirm the previous IHC study (Sivaparvathi et al., 1995) which showed more frequent and intense immunostaining for Cat B in more malignant forms of brain tumors. Similarly, another group (Mikkelsen et al., 1995) found the highest Cat B IHC score in GBM, compared with anaplastic astrocytoma and normal brain. Moreover, both groups reported heterogeneity in the staining intensity and its regional distribution, with the proliferative tumor margin staining more intensely than the tumor core. Researchers (Rempel et al., 1994) also observed altered subcellular localization of Cat B. They found Cat B expression to correlate with increased histological and radiological evidence of invasion (Rempel et al., 1994), which is consistent with the strong association of Cat B with the clinical and histological paramaters, indicating advanced tumors in our study. There is, thus, in general agreement that brain tumor progression is associated with increased expression of Cat B in tumor cells (Strojnik et al., 1999). Cat B immunostaining in proliferative endothelial cells was first reported by Mikkelsen et al., 1995, although Cat B immunostaining was lower in

endothelial than in tumor cells, as we observed in benign tumors. However, endothelial cell-associated Cat B immunostaining was present in about two-thirds of malignant tumors, compared with less than one-tenth of the benign tumors. Different models of in vivo angiogenesis have been proposed (Paku, 1998) and, according to them, one may speculate that Cat B actively participates in the intracellular lumen formation within the endothelial cell and/or that the secreted forms of Cat B directly degrade the ECM proteins (Buck et al., 1992; Liotta et al., 1991). Irrespective of the mechanism, our data implicate Cat B with brain tumor-induced angiogenesis.

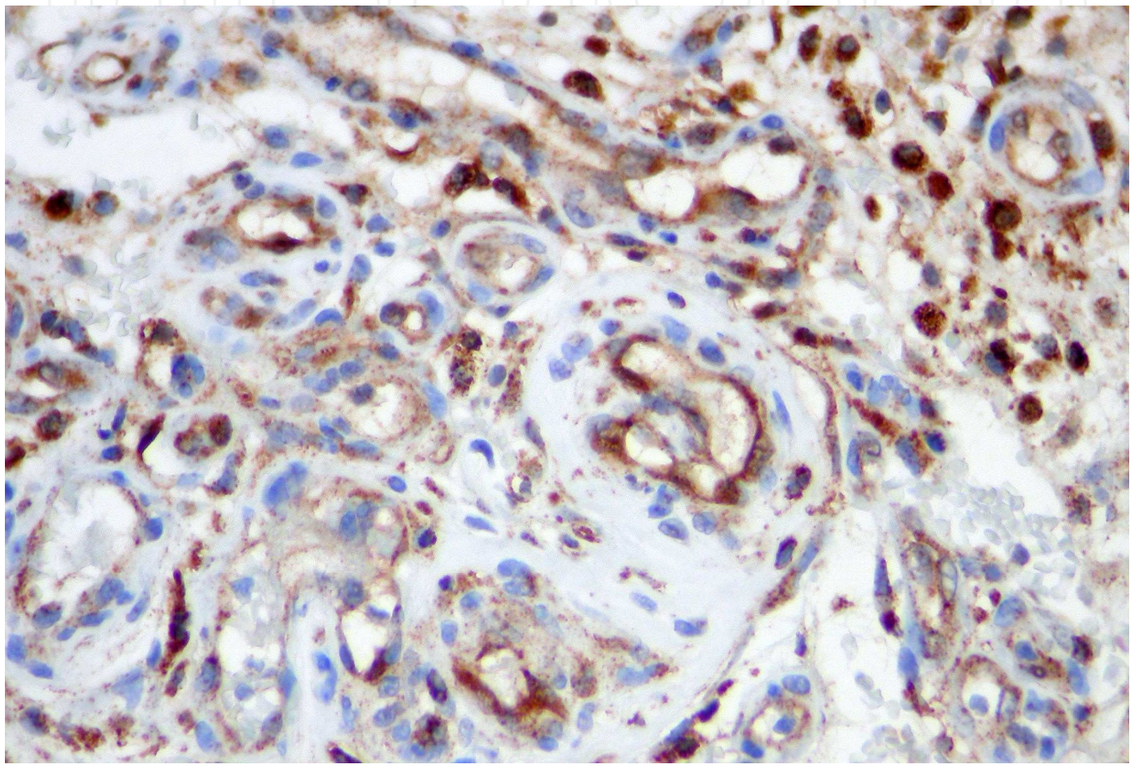


Fig. 7. Glioblastoma multiforme. Cat B antibody shows positively stained tumor cells and strong positive staining in endothelial cells (x40)

Our study (Strojnik et al., 1999) is the first clinical study on prognostic impact of Cat B in tumors of the central nervous system (CNS) and shows that the survival time is significantly longer in patients with low total immunostaining score, as compared with patients with strong staining. Intense Cat B staining of endothelial cells is prognostically important in patients with glioblastoma, indicating significantly shorter survival.

To summarize, we have demonstrated that immunostaining of cathepsin B correlated with high histological score and was significantly associated with poor clinical symptoms. The level of expression of cathepsin B in tumor and endothelial cells is a strong prognostic marker for primary tumors of the CNS. Intense immunostaining of cathepsin B in endothelial cells may be used to predict the survival of glioblastoma patients and, in addition, it indicates the involvement of cathepsin B in tumor-associated angiogenesis. These results suggest that the therapeutic application of cystein proteinase inhibitors should be targeted to both tumor and endothelial cells.

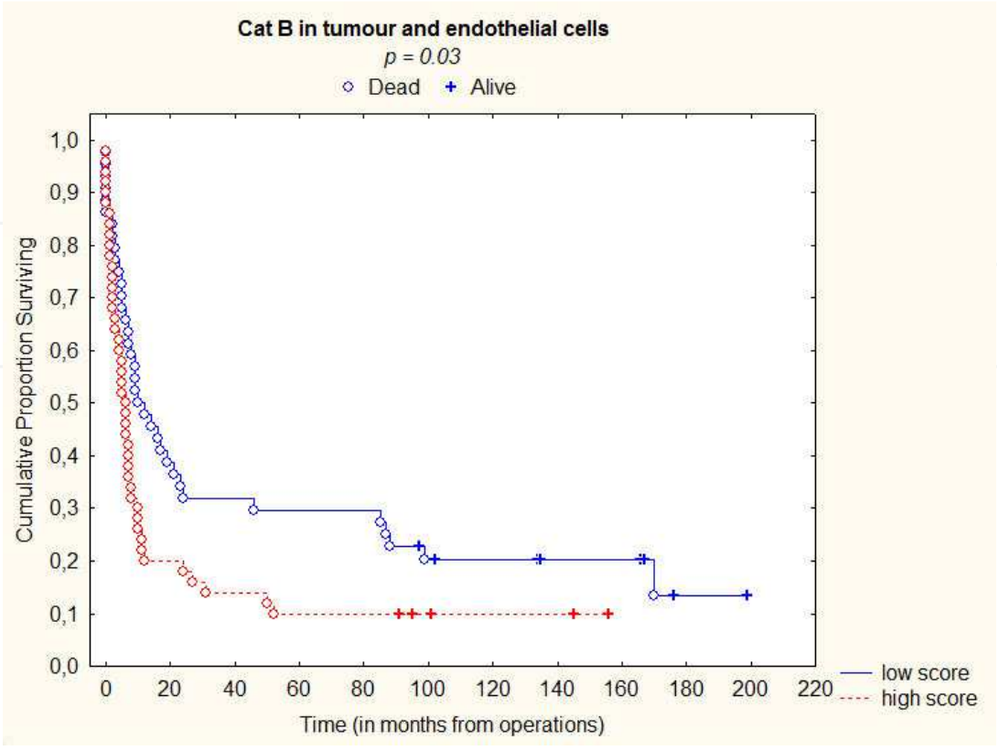


Fig. 8. Cat B in tumor and endothelial cells, and survival in all tumors

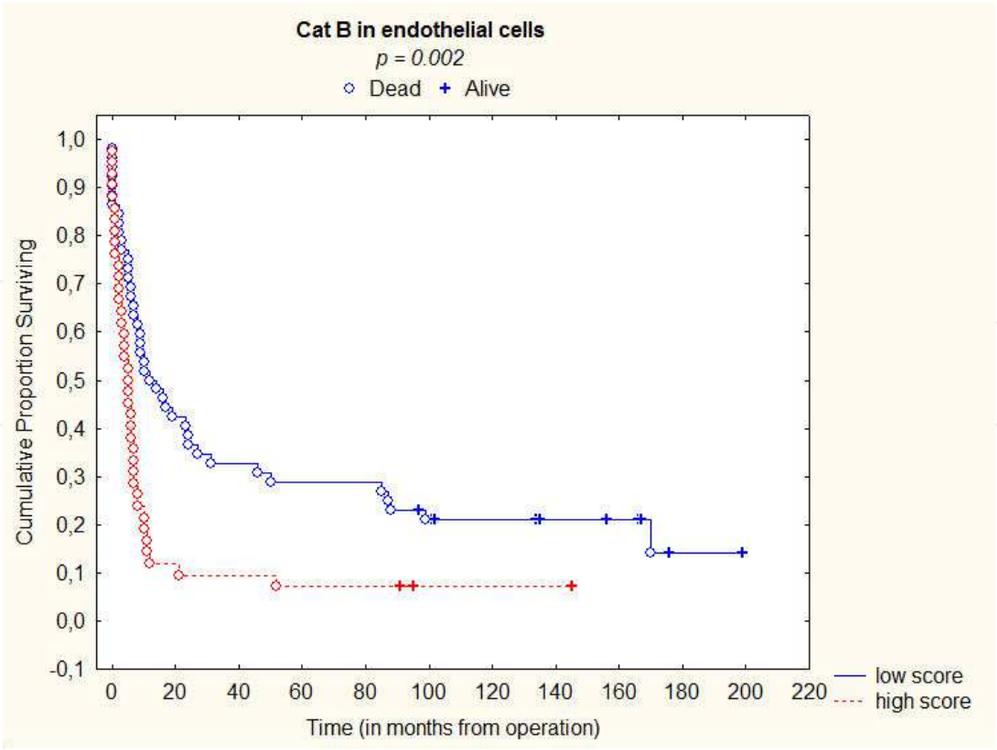


Fig. 9. Cat B in endothelial cells, and survival in glioblastoma multiforme

To complement our work we measured the expression of cathepsin L in the same population of human glioma patients (Strojnik et al., 2005). Cat L expression is also elevated in various types of human tumors (Lah & Kos, 1998) and its levels were prognostic for survival of patients with breast carcinoma (Thomssen et al., 1995). The only previous study of Cat L in brain tumor demonstrated that Cat L expression and activity correlated positively with increased malignancy of human glioma (Sivaparvathi et al., 1996b). We (Strojnik et al., 2005) have confirmed that in gliomas, cathepsin L is found predominantly in the malignant tumor cells. Cat B staining was expressed in both tumor and endothelial cells to the same extent. In contrast, Cat L was expressed significantly more in tumor cells than in the endothelilal cells. Although Cat L concentrations in all tumors appear to be lower than Cat B, we found significantly higher expression of Cat L in malignant than in benign gliomas. This implies a role for Cat L in malignant transformation of brain tumor cells. We also confirmed the correlation between immunostaining of Cat L in tumor cells and the histological score, i.e. the stage of glioma malignancy. Cat L and Cat B IHC staining correlated significantly. However, in contrast to Cat B (Strojnik et al., 1999), another study (Strojnik et al., 2005) did not reveal any prognostic value of Cat L, either in tumor or in endothelial cells.

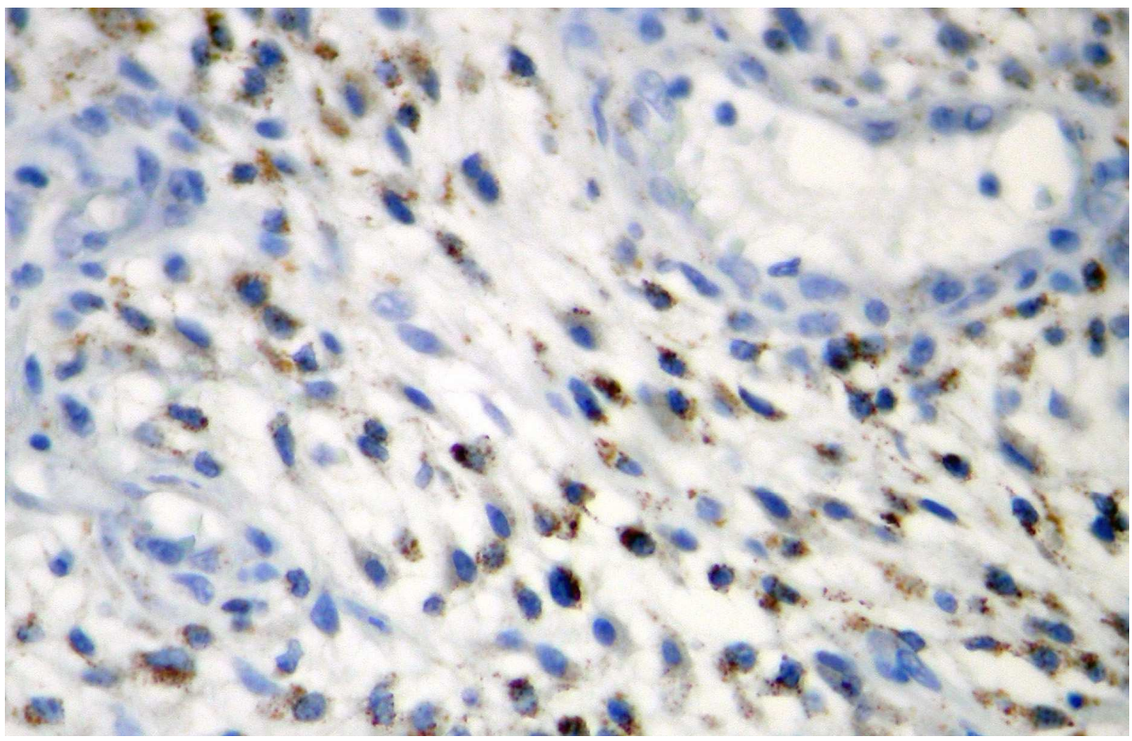


Fig. 10. Immunohistochemical staining of cathepsin L in glioblastoma multiforme (x40); Cat L antibody shows strong positive immunostaining in tumor cells but no staining in endothelial cells

In conclusion, Cat L is preferentially expressed in tumor cells, increasing with glioma progression, but is not significantly associated with new vasculature of glioblastoma. In contrast to Cat B, Cat L has no prognostic impact, suggesting different roles of the two cathepsins in glioma progression.

2.2.2 Neural stem cell markers nestin and musashi proteins

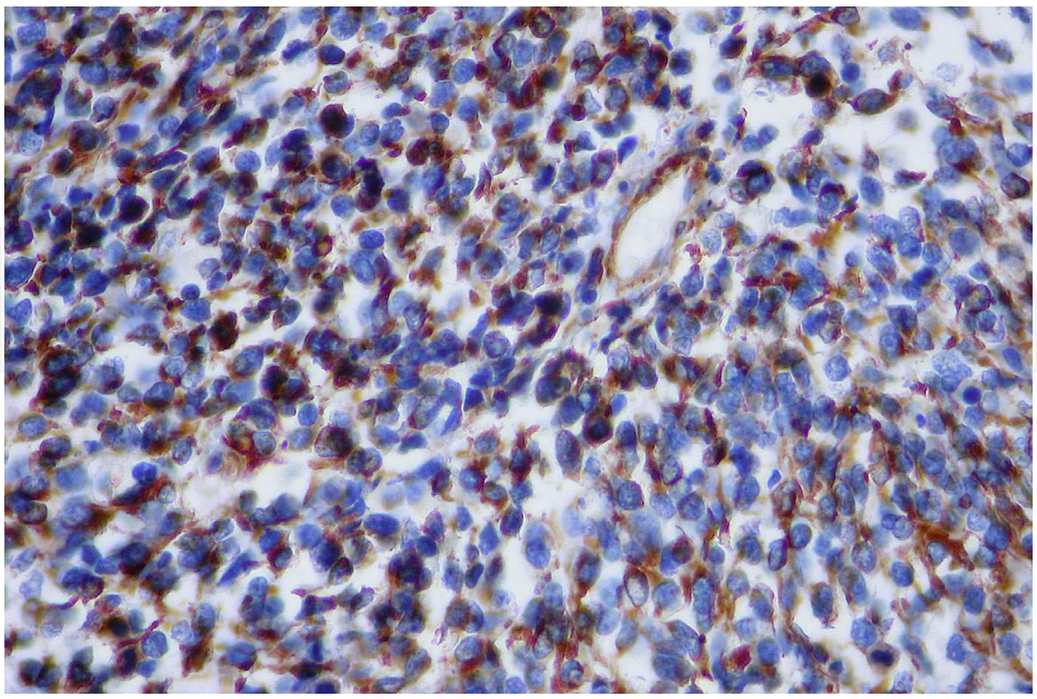
To identify other possible biological markers we studied nestin and musashi proteins, which are expressed by neural progenitor cells during CNS development. Nestin is an intermediate filament (IF) protein involved in the organization of the cytoskeleton, but it has also been implicated in cell signaling, organogenesis, and cell metabolism (Fuchs & Weber, 1994). Class VI IF nestin is expressed abundantly during early embryogenesis in neuroepithelial stem cells but is absent in most cells of the mature CNS (Lendahl et al., 1990). Nestin is down-regulated in mature cells (Steinert & Liem, 1990). It may be expressed in astrocytes of the adult CNS in response to cellular stress, such as neoplastic transformation (Dahlstrand et al., 1992; Tohyama et al., 1992). Nestin has been detected in primary CNS tumor but not in carcinoma metastases (Dahlstrand et al., 1992; Ikota et al., 2006).

The musashi family is an evolutionarily conserved group of neural RNA-binding proteins (Okano et al., 2002, as cited in Strojnik et al., 2007). Musashi 1 is selectively expressed in neural progenitor cells, including neural stem cells (Kaneko et al., 2000, as cited in Strojnik et al., 2007). Its expression is down-regulated with the progression of neurogenesis (Toda et al., 2001). The aim of our study (Strojnik et al., 2007) was to estimate the levels of nestin and musashi in tumor and endothelial cells of low- and high-grade gliomas with a view to correlate the levels to the histopathological score. A comparison was also made to the other prognostic markers, Cat B and L.

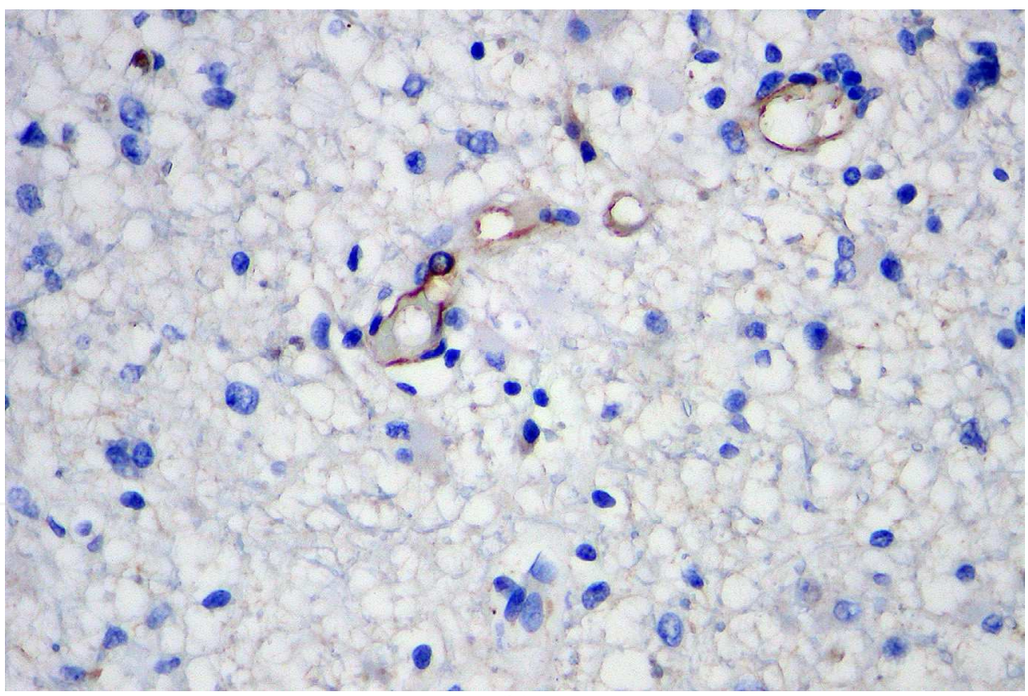
Recently, it has been reported that the immunohistochemical detection of nestin expression could be used as indicator of dedifferentiation and progression in astrocytomas (Ehrmann et al., 2005). Nestin is a useful marker for examining the infiltration of malignant cells into surrounding tissue (Kitai et al., 2010). In our study tumor cells from low-grade astrocytomas contain low levels of nestin and most high-grade gliomas express high levels of nestin (Strojnik et al., 2007).

It was proposed that nestin plays a role in tumor invasion of melanomas (Florenes et al., 1994, as cited in Strojnik et al., 2007). Nestin may therefore correlate with other markers of invasiveness, such as cysteine cathepsins B and L, which are both highly up-regulated in high-grade gliomas (Lah et al., 2000; Levičar et al., 2002; Sivaparvathi et al, 1995). In our study, a positive correlation between Cat B and nestin was established in high-grade gliomas, both markers correlating with the malignancy, as defined by histological scores. The exact location of nestin-positive cells was determined by mapping the distribution of nestin in highly invasive human glioma xenograft model. IHC staining of nestin in a xenograft model showed that nestin-positive cells are more abundant at the transition zone of the tumor, as reported for Cat B (Mikkelsen et al., 1995; Rempel et al., 1994). Given that invasion is associated with poor prognostic outcome for glioma patients, it is not surprising that nestin proved a very strong prognostic factor.

It is therefore reasonable to suggest that the switch to malignancy is associated with a significant increase of neural progenitor markers and with high expression of the invasive marker Cat B. Nestin expression correlated with lysosomal Cat L, which has been shown to be associated with glioma cell invasiveness but was not prognostic in our population of patients. This may be due to other biologic functions of Cat L associated, for example, with the cell cycle (Levičar et al., 2003) that may be less favorable for tumor progression. By multivariate Cox regression analysis, only nestin remained a good prognosticator of following variables: patients' age, sex, immunohistochemical scores in tumor and endothelial cells for nestin, cathepsin B and cathepsin L. In previous study (Strojnik et al., 1999), only univariate analysis was performed for Cat B staining in tumor cells, which



(a)



(b)

Fig. 11. Immunohistochemical staining of nestin protein in tumor and endothelial cells in glioblastoma (a) and astrocytoma (b). Intense staining of nestin is seen in the cytoplasm of tumors cells and is more abundant in the high (a) than in the low-grade (b) tumors. The IHC staining of nestin in endothelial cells was the same in the high- and low-grade groups.

showed prognostic significance ($p < 0.03$) but increased to $p < 0.003$ when both tumor and endothelial cells were considered. In multivariate analysis, it is not unusual, that one prognosticator falls out of the significance limits when compared simultaneously with much stronger prognosticator – in our case, the nestin.

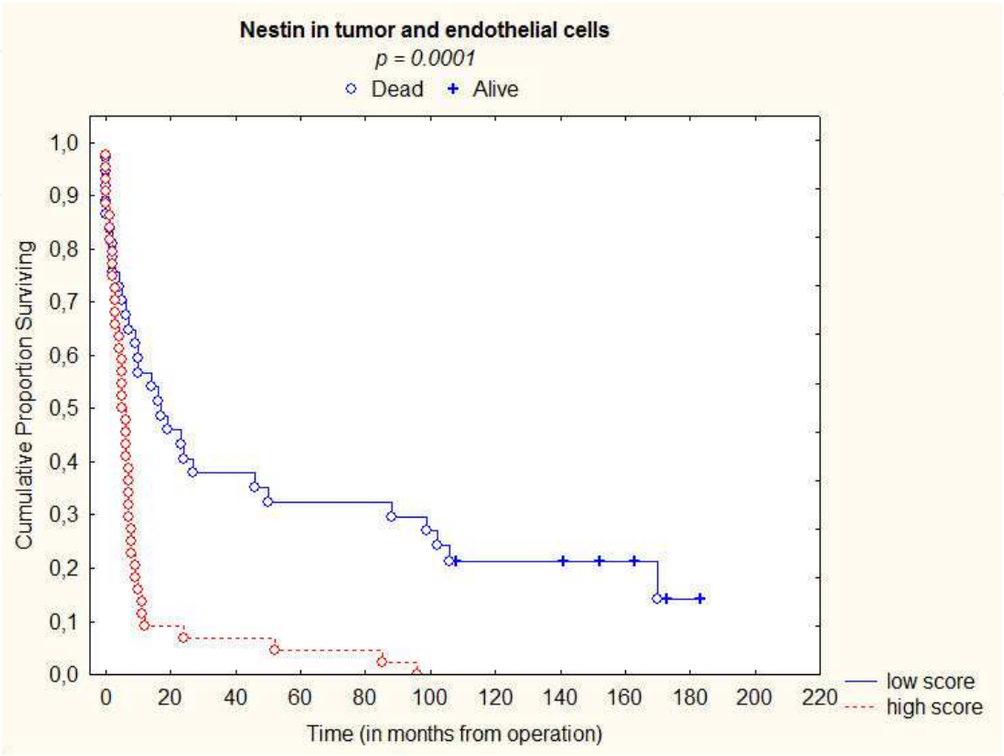
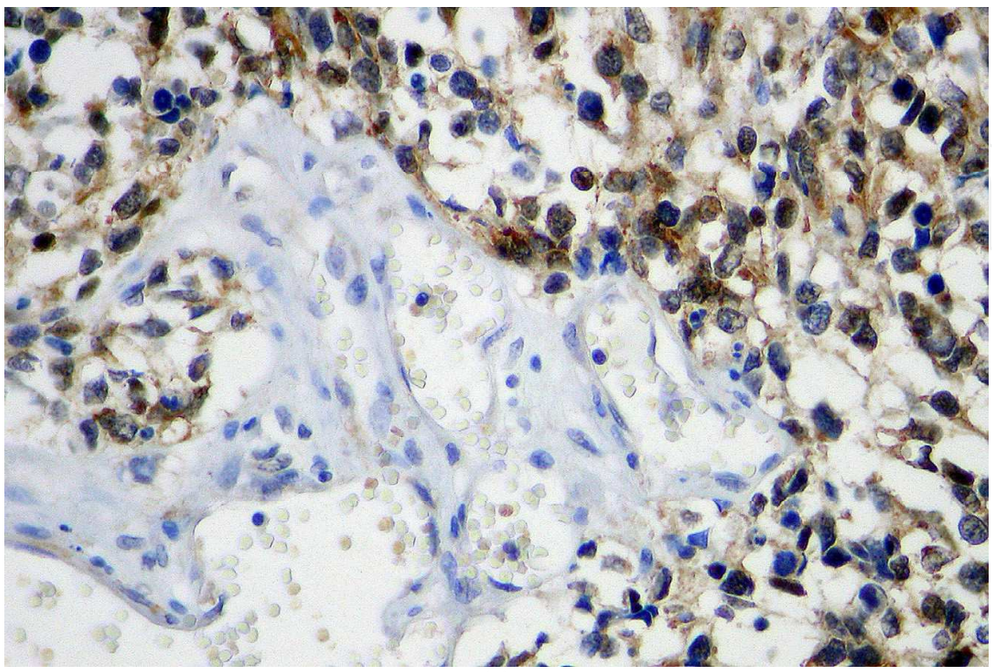


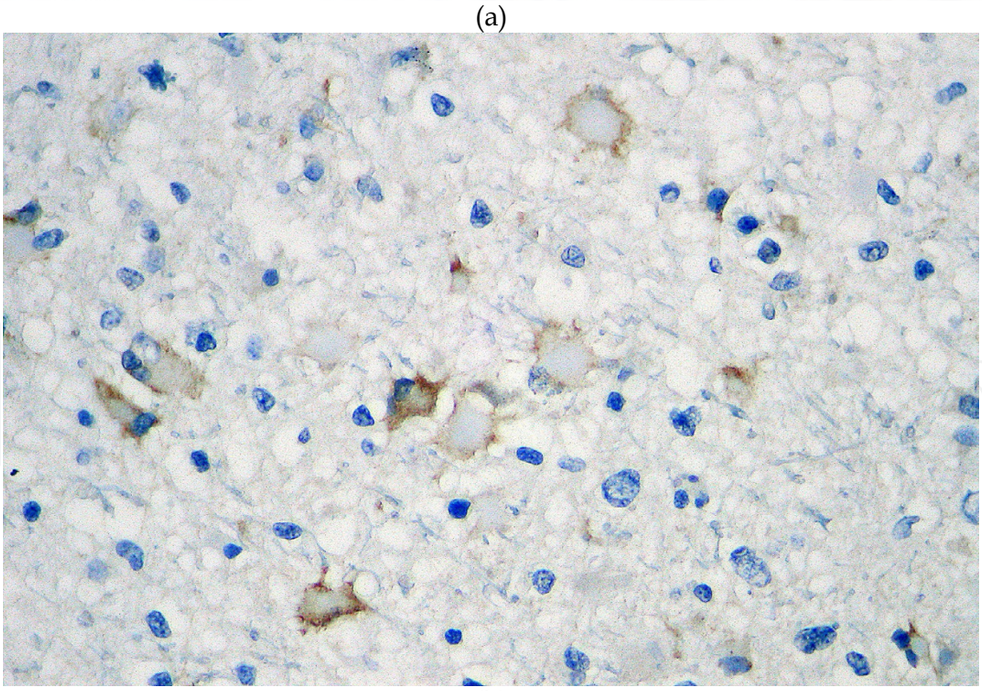
Fig. 12. Survival analyses. Patients stratified according to the median IHC score for nestin (high score or low score) in tumor and endothelial cells combined.

We have shown that the musashi protein is expressed, to a higher extent, in high-grade tumor cells than in those in the low-grade tumors. The expression of musashi was significantly weaker than that of nestin in tumor cells of the high-grade tumors (Strojnik et al., 2007). This differential expression pattern of the two neural progenitor cell markers in gliomas suggests that these two proteins may be stopped early (musashi) and late (nestin) in the BTSC "dedifferentiation" during GBM progression. Musashi is a marker of asymmetric cell division and is probably expressed as an early marker in stem/progenitor cell development. The fact that the musashi expression did not correlate with any prognostic factors may be related to the transformation process where the regulatory function of musashi is lost or changed. The difference in the prognostic impact of both stem cell markers is related to their different functions also. We hypothesize that these functions may be related to the invasiveness of possible stem/progenitor cell subpopulations. This is based on the observations that cathepsins, particularly Cat B – also the marker of glioblastoma cell invasiveness – strongly correlated with the nestin but not with the musashi expression.
It is noteworthy that Cat B and, to a lesser extent, Cat L, as well as nestin, were present in the endothelial cells. The cathepsins have been suggested to play a role in angiogenesis, possibly also assisting endothelial cell invasion during capillary formation (Caserman & Lah, 2004),

particularly in GBM (Mikkelsen et al., 1995; Strojnik et al., 1999). Expression of Cat B, but not Cat L, in endothelial cells of high-grade tumors is significantly higher than in low-grade astrocytomas and can be used as a prognostic factor for GBM patients (Strojnik et al., 2005).



(a)



(b)

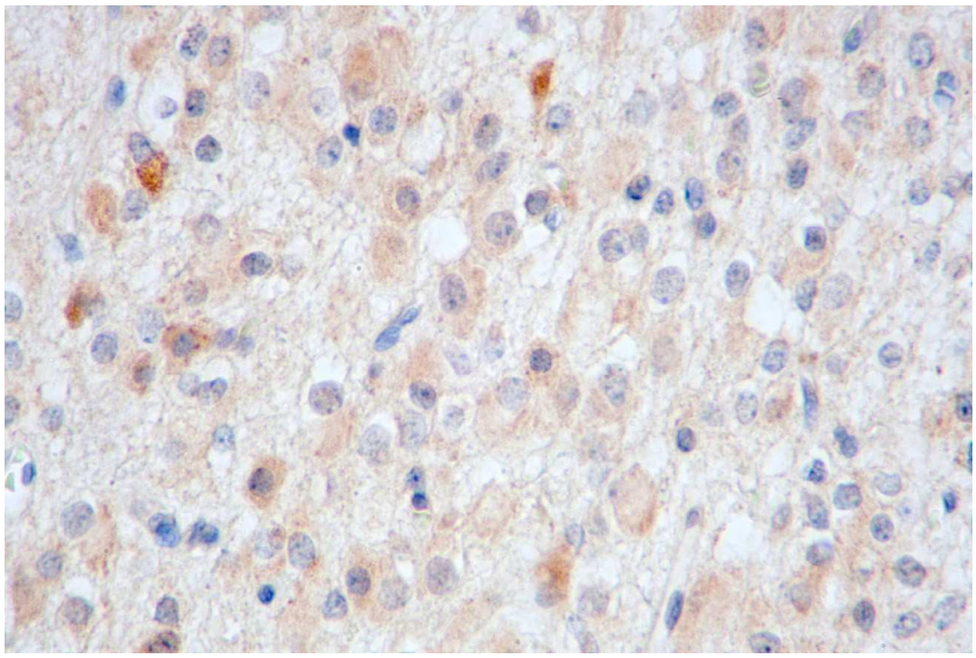
Fig. 13. Immunohistochemical staining of musashi protein in tumor and endothelial cells in glioblastoma (a) and astrocytoma (b). Musashi immunostaining was weak in endothelial cells of high-grade tumors.

Tumor angiogenesis may be initiated by various mechanisms, for example, "bursting" of new capillaries from the established blood vessels and by attracting angiopoietic stem cells from bone marrow. All the repertoire of protein markers for these stem cells is not known. As the musashi and nestin may be also the markers for the cancer-associated angiopoietic stem cells, we have also paid the attention to the fact that nestin, but, to a much lesser extent, the musashi, was indeed expressed in the endothelial cells. We showed that nestin was expressed in endothelial cells in both low- and high-grade tumors, whereas musashi was expressed only to a limited extent in endothelial cells in the high-grade tumors. We have therefore also estimated their potential impact on the prognosis. The latter was not found significant. However, the further research should confirm the hypothesis derived from our data, that is, that angiogenesis also may result predominantly from the bone marrow stem cells attracted to and differentiating onto blood vessels within the tumor.
On summary, we have confirmed that nestin is expressed in tumor cells and in endothelial cells of primary gliomas to a greater extent than musashi. In our clinical study on the prognostic impact of the neural progenitor cell markers nestin and musashi in tumors of the CNS, the high level of nestin, but not musashi, in tumor cells indicates significantly shorter survival of glioma patients. Intense immunostaining of nestin in tumor cells may be used to predict the risk of death in patients with malignant primary tumors of the CNS. Moreover, by multivariate analysis, nestin presents as the best prognosticator of all the variables. Our data links the invasive glioma cells to CNS precursor cells, indicating that the most malignant cells in the gliomas may well be closely related to the glioma stem cells.

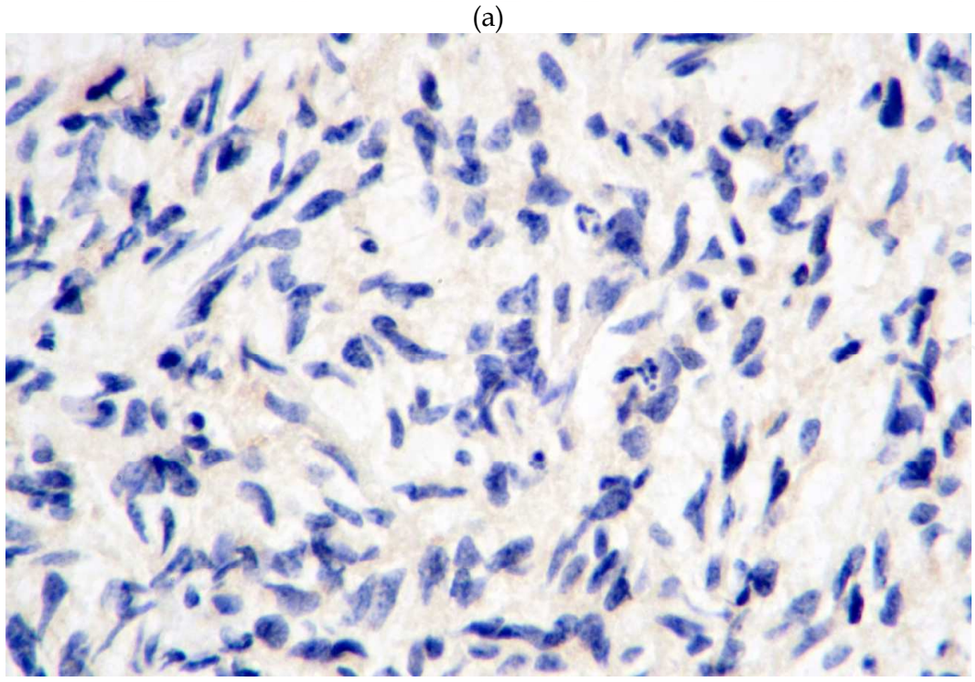
2.2.3 Expression of kallikrein 6 and CD68 in human gliomas

Subsequent study (Strojnik et al., 2009) was designed to evaluate the expression of kallikrein 6 and CD68 in human glioma, and investigate their prognostic significance for survival of brain cancer patients in comparison to previous established prognostic markers.
Kallikreins are expressed by secretory epithelial cells of many organs and have been implicated in a range of normal physiological functions. Some of the kallikreins are known for their clinical application as cancer biomarkers: for example kallikrein 3, which is known as prostate-specific antigen (PSA), is one of the best available biomarkers for monitoring tumor burden in sera of prostate cancer patients and has also been evaluated as a marker for prostate cancer diagnosis and prognosis (Loeb & Catalona, 2007). In vitro data and animal experiments have indicated the role of kallikreins in several steps of tumor progression, such as tumor growth, invasion and metastasis, as well as increased angiogenesis (Borgoño et al., 2004; Borgoño & Diamandis, 2004; Lundwall et al., 2006).
Kallikreins are also widely distributed in various areas of the human brain (Raidoo et al., 1996). It has been suggested that the functional role of kallikreins is to assist in the normal turnover of brain proteins and the processing of peptide hormones, neurotransmitters and nerve growth factors that are essential for normal neuronal function and synaptic transmission (Borgoño et al., 2004; Borgoño & Diamandis, 2004). Kallikrein 6 is among the members of the kallikrein family with high levels of expression in the brain and related fluids (Yousef at al., 2003); however, knowledge of the expression of kallikreins by brain tumors is limited. The objective of our study (Strojnik et al., 2009) was to evaluate the possible prognostic impact of serine protease kallikrein 6 in gliomas. Kallikrein genes/proteins are aberrantly expressed in many cancer types and they may exert diverse and often contrasting effects on the tumor and its microenvironment. Therefore, high kallikrein expression has been associated with either poor or favorable patient prognosis.

Some members of the kallikrein family are listed among the group of tumor-protecting proteases (Lopez-Otin & Matrisian, 2007), but even a single kallikrein may have a dual role in tumor progression. We (Strojnik et al., 2009) demonstrated higher IHC expression of kallikrein 6 in tumor cells of benign human gliomas comparing to malignant tumors.



(a)



(b)

Fig. 14. Immunohistochemical staining of astrocytoma with kallikrein 6 antibody revealed strong immunostaining in tumor cells (a) while in glioblastoma staining was weaker (b)

Our data support a potential role of kallikrein 6 in suppression of glioma progression, however, a prognostic value of kallikrein 6 was not revealed by our study. The possibility of a dual role (pro- and antitumor) of kallikrein 6 in tumor growth cannot be overlooked. Clinical studies with larger patient populations are needed to allow further evaluation of kallikrein 6 function in glioma progression.

CD68 is a transmembrane glycoprotein, expressed by monocyte/macrophage lineages and serves as a marker for microglia (Hulette et al., 1992). Microglial cells function as resident immune cells and phagocytes in the CNS. In response to pathology, resident microglia follows a stereotyped pattern of first becoming activated and then phagocytic (Trapp & Herrup, 2004). On one hand, microglia may represent components of the antitumor immune response in the CNS, which is inactivated by local secretion of immunosuppressive factors by glioma cells. On the other hand, taking into account that microglia are capable of secreting a variety of immunomodulatory cytokines, they may be attracted by the gliomas to assist in tumor growth (Badie & Schartner, 2001; Graeber et al., 2002). In human glioma, intratumoral microglia density is higher than in peritumoral and normal brain, and microglia increase in number according to grade of malignancy (Roggendorf et al., 1996; Morris & Esiri, 1991). It has been evidenced that microglia accumulation in diffuse glial tumors does not merely represent a nonspecific reaction to tissue injury but reflects participation of these cells in supporting and promoting the invasive phenotype of astrocytoma cells (Bettinger et al., 2002).

Notably, tumor cells can occasionally be reactive to some macrophage markers (Leenstra et al., 1995). They investigated six specimens of cultured astrocytoma cells and reported that nine macrophage markers, including CD68, were clearly reactive in neoplastic astrocytes, whereas astrocytes in normal brain specimens were not reactive (Leenstra et al., 1995). This study suggested that the demonstration of macrophages within astrocytoma by using macrophage-specific antibodies alone must be cautiously considered. In accordance with quoted studies, we also found strong CD68 expression in tumor cells of U87 human glioblastoma cell suspension, in U87 spheroids (both prepared from the U87 human glioblastoma cell line without microglia), as well as in induced rat tumors (Strojnik et al., 2006). In our CD68 study (Strojnik et al., 2009) we considered the possibility that human malignant astrocytes may adopt a macrophage phenotype and aimed to evaluate the possible prognostic value of CD68 expression for the survival of brain tumor patients. Both microglia and tumor cells expressed CD68. High CD68 staining score was significantly more frequent in the malignant than in the benign tumors.

We evaluated the antigen expression of CD68 in glioma tissue by avoiding any region with necrosis and excluding foamy cells, possibly indicating the presence of macrophage. However, malignant astrocytoma cells were also highly CD68 positive, in accordance with another report (Leenstra et al., 1995). These authors further emphasized that there may be biological properties shared by macrophages and astrocytoma cells, such as phagocytosis and production of the same growth and angiogenic factors. These common properties may be explained: (a) by genetic alterations during malignant transformation of astrocytes; (b) by fusion of astrocytes with macrophages; or (c) by another, as yet unknown, mechanism of gene transfer during glioma progression (Leenstra et al., 1995). These may lead to significantly higher CD68 immunostaining of the malignant glioma.

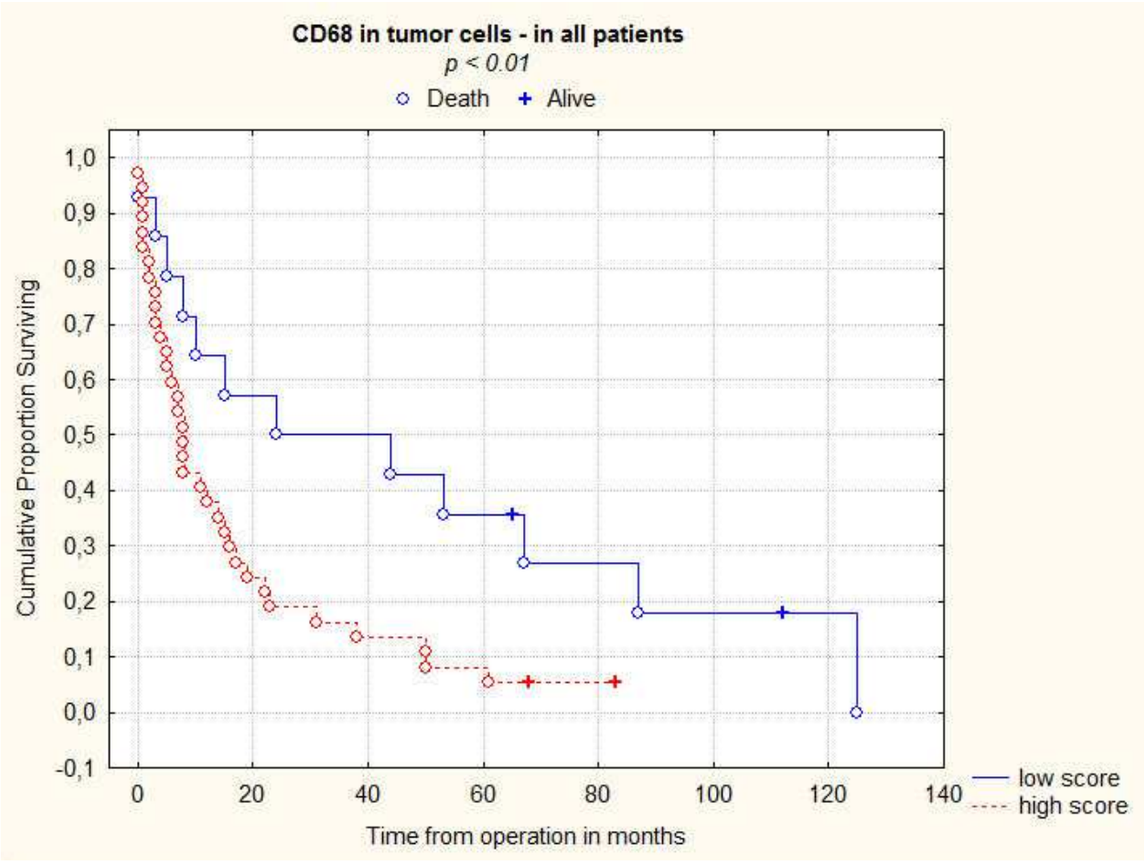
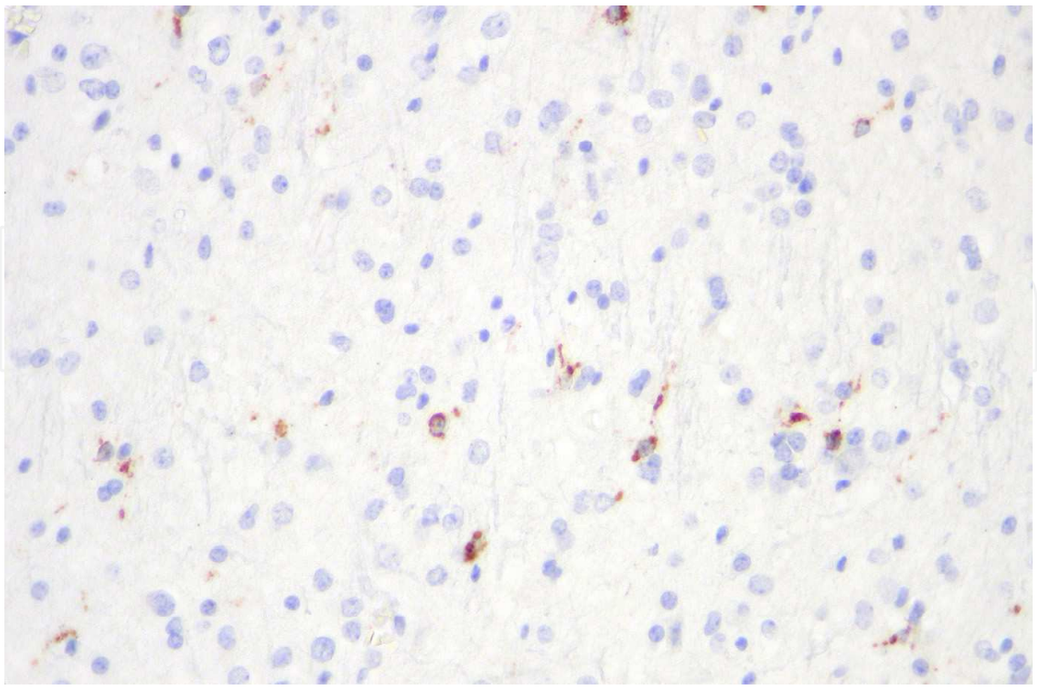


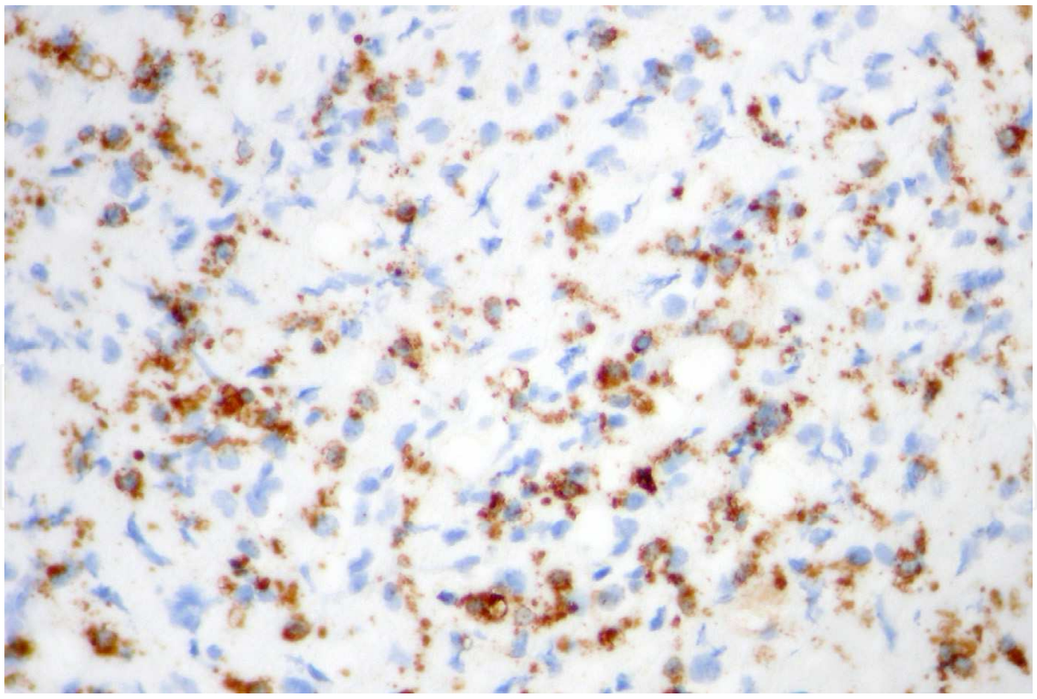
Fig. 15. Prognostic significance of immunolabeling for CD68 (low and high IHC score) for the survival of all patients with primary brain tumors

Survival analysis revealed that CD68 tumor staining has a prognostic value for glioma patients, comparable to that of cathepsin B. Within the malignant group, intense CD68 staining was marginally significant prognosticator for shorter survival. Notably, there was a significant prognostic value of CD68 tumor staining in the group of patients with anaplastic astrocytoma, which may be important for the management of patients with longer survival than these with glioblastoma. Further studies are necessary to investigate the possible mechanisms and consequence of macrophage phenotype expression of malignant astrocytomas, as well as possible role of microglia for tumor progression and patient prognosis.

To resume, kallikrein 6 was down-regulated in malignant glioma, but this differential expression did not have an impact on patient prognosis. In contrast, immunostaining of glioma tissue for CD68, as well as for Cat B and nestin, may be used as prognostic marker for survival of glioma patients. This finding suggests that besides the known role of Cat B in invasion and angiogenesis, nestin and CD68 may be also associated with glioma progression (Strojnik et al., 2007; Strojnik et al., 2009).



(a)



(b)

Fig. 16. Treatment of astrocytoma (x40) with CD68 antibody resulted in a few positively stained tumor cells (a); immunohistchemical staining of CD68 in glioblastoma multiforme (x40) revealed strong reaction in almost all tumor cells (b)

3. Conclusion

In recent years there have been many publications in the area of immunohistochemistry in brain tumor pathology (Takei et al., 2007; Dunbar & Yachnis, 2010; Ikota et al, 2006). Extensive molecular studies have identified diagnostic and prognostic markers in gliomas (Labussiere et al., 2010). They can assist in diagnosis, provide prognostic information and potentially predict response to therapy (Rivera & Pelloski, 2010). As we mentioned above, we combined the clinical work by different experimental glioma models. Animal tumor sections were examined for tumor markers by routine haematoxylin and eosin staining and immunohistochemical analyses. Established animal models provide a basis for further experimental studies of genetic and protein expression fingerprints during human glioma tumorigenesis. Furthermore, in a present paper we reviewed clinical and experimental work in glioma patients operated at our department of neurosurgery. Immunohistochemical studies supplement conventional H&E histology. Immunohistochemically we evaluated the expression of possible biological markers in human gliomas, including proteolytic enzymes (cathepsins B and L), neural stem cell markers (nestin, musashi), marker for microglia (CD68) and others (e.g. kallikrein 6). Differently from other reports, we performed immunohistochemical staining for the panel of markers on the same group of patients. Increased expression of lysosomal cystein proteinases such as cathepsins B and L plays a functional role in tumor cell migration and metastasis (Lah et al., 2000). We found that Cat B expression was highly elevated in GBM compared to lower grade malignant tumors and benign tumors. Cat B was also highly expressed in the endothelial cells of about two third of GBM. The latter finding indicates that Cat B may be associated with the invasion of not only tumor but also endothelial cells in the process of angiogenesis. At the end of the nineties we first published the clinical study on prognostic impact of Cat B in tumors of CNS, revealing that survival time in all patients with weak total immunostaining score is significantly longer compared to survival of patients with strong staining. Intense Cat B staining of endothelial cells is also prognostic important in patients with glioblastoma indicating significantly shorter survival. Cat L is preferentially expressed in tumor cells, increasing with glioma progression, but is not significantly associated with new vasculature of glioblastoma. Nestin is expressed in tumor cells of primary gliomas to a greater extent than musashi. Nestin-positive tumor cells are localized more abundantly in the transition zone of the tumor. Nestin is expressed in the endothelial cells in both low- and high-grade tumors, whereas musashi is expressed only to a limited extent in endothelial cells in the high-grade tumors. The further research should confirm the hypothesis derived from our data, that is, that angiogenesis also may result predominantly from the bone marrow stem cells attracted to and differentiating onto blood vessels within the tumor. Nestin is shown to be a strong prognostic marker for glioma malignancy. Our study revealed that both microglia and tumor cells expressed CD68. Malignant astrocytoma cells were highly CD68 positive in accordance to previous report (Leenstra et al., 1995). We found that some authors recommended to identify macrophages on intraoperative consulatiton to distinguish a neoplastic process from a demyelinating (or other destructive noneoplastic) disorder using IHC staining for CD68 (Dunbar & Yachnis, 2010). We point out that the demonstration of macrophages within the astrocytomas by using macrophage-specific antibodies alone must be cautiously considered. We further conclude that specific immunostaining of CD68 in tumor cells can be used to predict the risk of overall death in patients with glioma.

Noteworthy, we found prognostic value of CD68 immunostaining in tumor cells in anaplastic astrocytoma, which may be important for the management of patients with longer survival than these with GBM.

Taken together our data show that brain tumor progression is associated with increased expression of Cat B, Cat L, nestin and CD68 in tumor cells. We mentioned the role of Cat B and nestin in angiogenesis. IHC studies of biological markers provide important information about gliomagenesis. Biological marker can also assist in diagnosis. Immunohistochemistry has provided us with the ability to differentiate between tumors that are histologically indistinguishable. With this tool, we can now attribute the unpredictable behavior of what was once recognized as one tumor type to the more predictable behaviors of multiple tumors that are distinguishable based on their protein expression. The most challenged is to use findings for target therapy. Screening for the invasiveness of the individual brain tumor might help the neurosurgeon to define his strategy for further postoperative treatment of a given brain tumor.

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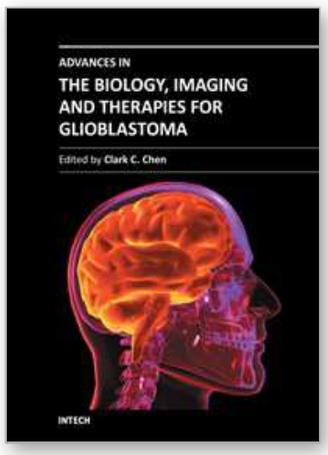
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