Cathepsin B Immunohistochemical Staining in Tumor and Endothelial Cells Is a New Prognostic Factor for Survival in Patients with Brain Tumors

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ABSTRACT
The cysteine endopeptidase, cathepsin (Cat) B, and its endogenous inhibitor, stefin A, were found relevant for cancer progression of many neoplasms, including human brain tumors. Histological sections of 100 primary brain tumors, 27 benign and 73 malignant, were stained immunohistochemically for Cat B and stefin A. The immunohistochemical staining of Cat B in tumor cells, endothelial cells, and macrophages was scored separately from 0–12. The score in tumor and endothelial cells was significantly higher in malignant tumors compared with benign tumors (P <0.000). A significant correlation between immunostaining of Cat B (scored together for tumor and endothelial cells) and clinical parameters, such as duration of symptoms, Karnofsky score, psycho-organic symptoms, and histological score was demonstrated. Univariate survival analysis indicated that total Cat B score above 8 was a significant predictor for shorter overall survival (P = 0.003). In glioblastoma multiforme, intense Cat B staining of endothelial cells was a significant predictor for shorter survival (P = 0.003). Stefain A immunostaining was weak and detected only in a few benign and some malignant tumors, suggesting that this inhibitor alone is not sufficient in balancing proteolytic activity of Cat B. We conclude that specific immunostaining of Cat B in tumor and endothelial cells can be used to predict the risk of death in patients with primary tumors of the central nervous system.

INTRODUCTION
Glomas are the most common form of brain tumors, contributing to more than half of the incidence of brain tumors (1). These tumors show a high degree of diffuse local invasion of normal nervous tissue (2–5). Tumor cells often infiltrate several millimeters beyond any obviously defined tumor margin, which prevents a complete surgical removal of malignant tissue and contributes to the high incidence of recurrence even after radical surgical resection (6–8).

The mechanisms underlying brain tumor cell invasion are not well understood. The process involves cell attachment to the ECM, motility, and degradation of ECM proteins by proteolytic enzymes (9, 10). Previous studies on non-CNS tumors have demonstrated secretion of proteolytic enzymes and/or alterations in the regulation of proteolysis (11–16). 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 (17). Endopeptidases of four classes (serine, metallo-, aspartic, and cysteine), acting either alone or in linked, proteolytic cascade reactions, have been implicated in the biological mechanism of tumor progression for many types of cancer (18). Furthermore, Schmitt et al. (19) have suggested that these proteases are also useful new biological prognostic factors. Lysosomal enzymes, urokinase plasminogen activator (20), and certain types of matrix metalloproteinases also have been suggested to play a role in brain tumor progression (7, 21–25).

Lysosomal Cats comprise intracellular proteinases of different classes. Levels of aspartic Cat D (26, 27) and cysteine Cat B, Cat L, and Cat H (13, 28, 29) were found to be related to malignant progression and prognosis of carcinomas, such as breast (16, 26, 29–33), head and neck, (34) and colorectal carcinoma (35) and melanoma (36). Malignant progression of human gliomas was first found to be associated with an increase in cysteine proteases by McCormick et al. (14), and later by Sivaparvathi et al. (37), who demonstrated high levels of Cat B in tumor and endothelial cells of tumor tissue. Rempel et al. (38) demonstrated a large increase in Cat B expression during the malignant progression of human brain tumors. Increased protein and transcription levels were found in high-grade glioblastoma. Mikkelsen et al. (11) reported that Cat B levels in glial tumor cells correlated to the degree of malignancy of these cells.

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3 The abbreviations used are: ECM, extracellular matrix; ABC, avidin biotin-peroxidase complex; Cat, cathepsin; CNS, central nervous system; CP, cysteine proteinase; CPI, cysteine proteinase inhibitor; CT, computer tomography; IHC, immunohistochemical; MAB, monoclonal antibody.
Although Cat B has been found to be associated with tumor cells, macrophages, and endothelial cells, the prognostic value of the specific cellular expression of Cat for survival of brain tumor patients has not, thus far, been demonstrated.

CPs are regulated by endogenous inhibitors, comprising the large superfamily of cystatins, which is subdivided into three families: stefins, cystatins, and kininogens (39). Intracellularly, the most abundant inhibitors are stefin A and stefin B. Stefain A is mostly localized to epithelial and lymphoid tissue, whereas stefin B is uniformly distributed among tissues. It has been shown that rat brain tissue contains small amounts of cystatin α, the rat analogue of stefin A (40). Decreased IHC labeling of stefin A was reported in the dendritic reticulum cells of malignant lymphoid follicles (41), and lower levels of stefin A transcripts were found in breast cancer patients with advanced disease (42), compared with the respective normal tissues. In brain tumors, down-regulation of the total inhibitory activity of cystatins was observed and presumed to contribute to tumor malignancy (43).

The aim of the present study was: (a) to establish the cellular distribution of Cat B and stefin A by IHC staining in primary tumors of the CNS; (b) to correlate the expression of Cat B and stefin A with clinical, radiological, and histological features of brain tumors; and (c) to establish the possible prognostic value of these two biological markers for the disease outcome.

MATERIALS AND METHODS

 Patients. One hundred patients (53 male and 47 female) with primary tumors of the CNS, operated on at the Department of Neurosurgery, Maribor Teaching Hospital, between 1986 and 1996, were studied. The ages of the patients ranged from 3–75 years (median, 50.0). The histological slides of all cases were reviewed and classified according to the WHO classification of malignant tumors (44). There were 27 benign and 73 malignant tumors (Table 1). The median ages of patients with malignant and with benign tumors were 55 and 32 years, respectively.

Patient data included age, sex, date and type of initial operation, clinical neurological examination (Table 2), CT features (Table 3), data of adjuvant therapy (radiation, chemotherapy), and details of follow-up. Clinical parameters included: (a) duration of symptoms before the diagnosis of tumor; (b) psycho-organic changes; (c) neurological deficits; and (d) Karnofsky scale for assessing the functional state of the patient. Radiological parameters of the malignant tumor on brain CT scan included peritumor edema, necrosis, tumor border, midsagittal shift, hemorrhage, and enhancement with contrast medium (Table 3). Adjuvant therapy had been used in 56 patients. Among malignant tumors, 41 patients (56%) were treated by irradiation, 2 patients by additional chemotherapy, and 2 patients by the combination of irradiation, chemotherapy, and immunotherapy. In patients with benign tumors, 11 of 27 patients (41%) had been irradiated.

One neurosurgical resection of the tumor was performed in 88 of the patients, 11 patients were operated on twice, and 1 patient was operated on three times. In the subgroup of 12 patients with reoperations, five astrocytomas developed later into malignant tumors (two anaplastic astrocytomas and three glioblastomas). The remainder had a malignant tumor at the initial operation. The survival analysis included only primary tumors, but in the descriptive analysis of IHC staining we included the 11 cases of secondary tumors from the reoperated patients. The survival time was determined as the interval between initial operation and the patient’s death or January 1998, for those surviving to this date.

 Chemicals and Reagents. All chemicals in the study were of analytical grade. MAbs for immunohistological detection of Cat B and stefin A were from KRKA, d.d. (Ljubljana, Slovenia). They were obtained from 3E1 and C5/1 hybridoma cell lines, respectively, as described (12, 36). Briefly, hybridomas producing specific MAbs were grown in DMEM media containing 13% FCS to a high titer, followed by purification on a Protein G column (Pharmacia, Uppsala, Sweden). MAbs were characterized regarding specificity and applicability on cryostat and paraffin-embedded...
sections. Characteristics of the antibodies have been described previously (12, 35, 36).

**Histological Analysis.** A paraffin block with the most representative tumor tissue was selected, and 2–3-μm sections were cut for H&E and immunostaining. H&E-stained sections were scored by an independent pathologist. Tumors were classified according to the WHO classification (44). Five standard morphological features of malignancy were assessed: hypercellularity, nuclear pleomorphism, mitosis, vascular endothelial proliferation, and necrosis, each with 0, +, ++, and ++++. The histological score for one section was the sum of these five parameters and was placed in one of three subgroups: 1, 0 to 5; 2, 6+ to 10+; and 3, 11+ to 15+

**Immunohistochemical Analysis.** IHC staining was performed using the standard technique, according to the protocols of the Department of Pathology at the Institute of Oncology (Ljubljana, Slovenia). Sections (2–3-μm thick) were mounted on aminoethoxysilane-coated glass slides. The slides were dried overnight at 37°C and then at 57°C for 8 h. The slides were dewaxed in xylene, hydrated, and placed in 10 mM sodium citrate buffer (pH 6.0) and boiled for 6 min in a microwave (Miele M 752, 850 W) for antigen retrieval. Boiling was repeated in fresh buffer solution for another 10 min. Immunostaining was performed with an automatic immunostainer, TM 500 (DAKO-Bio Tek). The slides were incubated with anti-Cat B MAb clone 3E1 (1:100 dilution) and with antistefin A MAb (500 (DAKO-Bio Tek). The slides were incubated with anti-Cat B IHC score in tumor cells, endothelial cells, and macrophages. The frequency of Cat B and stefin A immunoreactivity in tissue sections was evaluated as negative when no positive cells were observed within the tumor, weak (1+) when <30% of the tumor cells were positive, moderate (2+) when 30–60% of the tumor cells were positive, and strong (3+) when >60% of tumor cells were positive. The intensity of staining was evaluated as 0, 1+, 2+, and 3+ for no staining, weak staining, medium staining, and strong staining, respectively. IHC score was determined as the sum of the frequency and intensity score for tumor cells, endothelial cells, and for both types of cells together. Immunostaining with CD68 antibody for identification of macrophages was used to differentiate between macrophage-associated Cat B and tumor cell-associated Cat B. The results of Cat B staining, in tumor cells and in endothelial cells, were subdivided into two groups with weaker staining (scored 0 to 4+) and with more intense staining (scored 5+ to 6+). The results of staining in both types of cells together were subdivided into groups with scores 0 to 8+ and 9+ to 12+ as weak and strong positive staining, respectively.

**Statistical Analysis.** Statistical analysis was performed using the program Statistica for Windows 5.1 (StatSoft, Inc.). Variables used in the analysis included histological score and Cat B IHC score in tumor cells, endothelial cells, and macrophages. Descriptive statistic methods were used to reveal Cat B association with the clinically and histologically different groups of patients.

Overall survival probabilities were calculated by the method of Kaplan-Meier (45). Log-rank test was used to assess the association between survival and the variables.

**RESULTS**

**Histological Analysis.** The histological parameters of tumors are summarized in Table 4. Morphological characteristics of malignancy were analyzed in 83 tumors (72 primary brain tumors and 11 secondary tumors from reoperated patients). In benign tumors, only one patient had a total histological score >5. In contrast, in the group of malignant tumors (primary and secondary), higher total scores were more frequent; seven percent scored from 0–5, 54% scored from 6–10, and 39% scored from 11–15.
Table 4  Histological parameters of tumors of the CNS

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Degree of abnormality</th>
<th>Benign tumors ( n = 27 ) (%)</th>
<th>Malignant tumors ( n = 83^a ) (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell density</td>
<td>1+</td>
<td>19 (70)</td>
<td>2 (2.5)</td>
</tr>
<tr>
<td></td>
<td>2+</td>
<td>8 (30)</td>
<td>60 (72.5)</td>
</tr>
<tr>
<td></td>
<td>3+</td>
<td>21 (25)</td>
<td></td>
</tr>
<tr>
<td>Pleomorphism</td>
<td>0</td>
<td>2 (7)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1+</td>
<td>22 (81)</td>
<td>1 (1)</td>
</tr>
<tr>
<td></td>
<td>2+</td>
<td>3 (12)</td>
<td>67 (81)</td>
</tr>
<tr>
<td></td>
<td>3+</td>
<td>15 (18)</td>
<td></td>
</tr>
<tr>
<td>Mitoses</td>
<td>0</td>
<td>23 (85)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1+</td>
<td>4 (15)</td>
<td>25 (30)</td>
</tr>
<tr>
<td></td>
<td>2+</td>
<td>12 (45)</td>
<td>32 (39)</td>
</tr>
<tr>
<td></td>
<td>3+</td>
<td>26 (31)</td>
<td></td>
</tr>
<tr>
<td>Endothelial cell proliferation</td>
<td>0</td>
<td>23 (85)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1+</td>
<td>12 (14)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2+</td>
<td>15 (37)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3+</td>
<td>17 (21)</td>
<td></td>
</tr>
<tr>
<td>Necrosis</td>
<td>0</td>
<td>27 (100)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1+</td>
<td>19 (23)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2+</td>
<td>7 (8)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>3+</td>
<td>32 (39)</td>
<td></td>
</tr>
<tr>
<td>Total histological score</td>
<td>0–5+</td>
<td>26 (96)</td>
<td>6 (7)</td>
</tr>
<tr>
<td></td>
<td>6+–10+</td>
<td>1 (4)</td>
<td>45 (54)</td>
</tr>
<tr>
<td></td>
<td>11+–15+</td>
<td>32 (39)</td>
<td></td>
</tr>
</tbody>
</table>

\( ^a \) Seventy-two cases from initial operation (one section missing) and 11 cases from reoperations.

Immunohistochemical Analysis. Cat B immunostaining was evaluated for 111 tumors (Table 5). The analysis included 100 cases of primary brain tumor and 11 cases from reoperations. There were 27 benign tumors, 73 malignant tumors from initial operation, and 11 malignant tumors from reoperations. Staining for Cat B in tumor cells from malignant tumors (\( n = 84 \)) was significantly more pronounced than in the benign tumors (\( n = 27 \)), both with respect to frequency (\( P = 0.03 \)) and intensity (\( P = 0.01 \)). Staining for Cat B in endothelial cells showed a similar pattern (\( P < 0.0001 \) for both). Staining for Cat B in tumor and endothelial cells combined was also significantly (\( P < 0.0001 \)) higher in malignant than in benign tumors (Fig. 1).

Immunostaining for stefin A was observed only in six cases (one astrocytoma, two medulloblastomas, and three glioblastomas multiforme) and then only in a few tumor cells (Fig. 2).

Correlation between Cat B Immunostaining and Clinical, Radiological, and Histological Parameters of Brain Tumors. Clinical parameters, such as duration of symptoms, psycho-organic changes, Karnofsky scale, and neurological deficit in cranial nerves, were significantly more pronounced in patients with malignant tumors (\( P < 0.01 \)). In all cases, a significant correlation was observed between clinical parameters and the overall IHC staining of Cat B in tumor and endothelial cells. For example, a significant (\( P < 0.05 \)) correlation of total Cat B score and the duration of symptoms, Karnofsky score, and psycho-organic changes, was observed. Cat B immunostaining in endothelial cells correlated well with neurological deficits in cranial nerves. Also, significant correlation (\( P < 0.01 \)) was observed with some of the radiological features, such as necrosis, edema, and with midsagittal shift. With respect to histological parameters, significant correlation (\( P = 0.005 \)) was found between the total Cat B immunohistochemical score (in tumor and endothelial cells) and the histological score in malignant tumors.

Prognostic Relevance of Clinical and Histological Parameters. In this study, the follow up varied from 0–135 months (median, 7). During this time, 78% of patients died (see Table 6). The rate of death was much lower in the group of benign tumors (45%; i.e., 12 patients) compared with the group of malignant tumors (90.5%; i.e., 66 patients), whereas 93% (54 patients) died within the group of patients with glioblastoma multiforme. Significantly (\( P < 0.0001 \)) shorter survival (median, 5 months) was observed in patients with malignant tumors compared with those with benign tumors (median, 11.5 months). A high histological score was highly significant (\( P < 0.0001 \)) for poor prognosis, as shown on Fig. 3.

Prognostic Relevance of Cat B. IHC analysis for total staining of Cat B by tumor and endothelial cells showed that patients with a score above 8 (9+ to 12+) had significantly (\( P = 0.003 \)) shorter survival times than patients with lower scores, as seen in Fig. 4. In the subgroup of malignant tumors (glioblastomas multiforme), no prognostic impact of tumor cell-associated Cat B by IHC analysis was observed. However, significant differences in survival of glioblastoma patients were observed between the groups of patients with low Cat B score (0 to 4+) and high Cat B score (5+ to 6+) in the endothelial cells alone (\( P = 0.003 \)), as shown in Fig. 5.

DISCUSSION

In this study of 27 benign and 84 malignant brain tumors, including 58 specimens of glioblastoma multiforme, we have shown that Cat B is expressed in glial tumor cells, proliferative endothelial cells, and macrophages near vessels adjacent to necrotic areas. Significantly more cases with high Cat B IHC score in tumor and endothelial cells were observed in malignant compared with benign tumors. In glioblastoma, most intense
staining was found in the majority (73%) of cases, although the majority of the benign tumors stained weakly. These results confirm the previous IHC study by Sivaparvathi et al. (37), who found more frequent and intense immunostaining for Cat B in more malignant forms of brain tumors. Similarly, Mikkelsen et al. (11) found the highest Cat B IHC score in glioblastoma multiforme, compared with anaplastic astrocytoma and normal brain. Moreover, both groups reported a heterogeneity in the
staining intensity and its regional distribution, with the proliferative tumor margin staining more intensely than the tumor core. Rempel et al. (38) also observed altered subcellular localization of Cat B, as suggested previously for some other types of tumors (46). They found Cat B expression to correlate with increased histological and radiological evidence of invasion (38), which is consistent with the strong association of Cat B with the clinical and histological parameters, indicating advanced tumors in the present study. There is, thus, general agreement that brain tumor progression is associated with increased expression of Cat B in tumor cells. A similar association has been observed for IHC score of Cat B in tumor cells of lung.

Fig. 2 IHC staining of primary brain tumors. A, glioblastoma multiforme stained with H&E (×100). B, glioblastoma multiforme. Same section stained with anti-Cat B antibody (×100). C, glioblastoma multiforme. Same section stained with stefin A antibody (×100). D, medulloblastoma. Tumor cells (arrow) stained by antistefin A antibody (×200). E, control section of the liver (arrow) stained with anti-Cat B antibody (×100). F, control section of skin epidermis (arrow) stained by stefin A antibody (×100).
(47), bladder (48), and colon carcinoma (49), but not in breast carcinoma (16).

Tumor-associated macrophages seem to play a role in tumor progression because they can either assist tumor cells in invasion and metastasis (50) or mediate cytotoxic effects to tumor cells (51). Increased expression of CPs has been observed in activated macrophages \textit{in vitro} (52). Macrophages are stained for Cat B and Cat L in intraductal breast carcinomas and for Cat B in lung carcinoma. However, in the present study, macrophage-associated Cat B expression had no bearing on prognosis and, therefore, the role of macrophage-associated Cat B in brain tumor progression, if any, still remains unknown.

Cat B immunostaining in proliferative endothelial cells was first reported by Mikkelsen et al. (11), 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. Cat B staining of new microvessels was recently observed in human prostate carcinoma (53). In breast carcinoma, Cat B labeled endothelial cells, but not blood vessel walls. Different models of \textit{in vivo} angiogenesis have been proposed (17) 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 (54, 55). Irrespective of the mechanism, our data implicate Cat B with brain tumor-induced angiogenesis.

This is the first clinical study on prognostic impact of Cat B in tumors of the 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. IHC analysis of tumor cell-associated Cat B has previously been shown to be useful for prognosis in lung (47) and colon (49) carcinoma patients.

\begin{table}
\centering
\begin{tabular}{|l|c|c|c|c|}
\hline
& All tumors & Benign tumors & Malignant tumors & Glioblastoma multiforme \\
& (n = 100) & (n = 27) & (n = 73) & (n = 58) \\
\hline
Alive at January 1998 & 19 & 15 (55%) & 4 (5.5%) & 1 (1.5%) \\
\hline
Time from operation (mo)* & 43 (18–135) & 72 (22–135) & 32 (18–39) & 28 \\
\hline
Dead & 78 & 12 (45%) & 66 (90.5%) & 54 (93%) \\
\hline
Time from operation (mo)* & 5.5 (0–99) & 11.5 (0–50) & 5 (0–99) & 5 (0–99) \\
\hline
Missing information & 3 & & 3 (4%) & 3 (5.5%) \\
\hline
\end{tabular}
\caption{Survival of patients}
\end{table}

\textsuperscript{*} Median time interval and total interval from the operation of primary tumor until January 1998.

\textsuperscript{a} Median time interval from the date of the operation until death.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig3}
\caption{Histological score and survival.}
\end{figure}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig4}
\caption{Cat B in tumor and endothelial cells, and survival in all tumors.}
\end{figure}


Stefin A is one of the intracellular inhibitors of CP, presumably associated with malignant progression of carcinomas and sarcomas (15, 28, 29). McCormick (14) reported that all gliomas in vitro secrete significant amounts of CPIs. Sivaparvathi et al. (43) reported that CPI activity was significantly higher in normal brain and in low-grade glioma compared with anaplastic astrocytoma and glioblastoma. In our tumor specimens, stefin A was present in minute amounts and was markedly lower than in skin control tissue and in breast tumor tissues (56). At present, it is not possible to speculate on the apparent down-regulation of stefin A in brain tumors because low levels were detected both in benign and in malignant tumors.

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

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