Cathepsin D Is a Potential Serum Marker for Poor Prognosis in Glioma Patients

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Abstract

Cathepsin D is an aspartyl protease involved in protein catabolism and tissue remodeling which can be secreted from cancer cells. To identify a potential serum marker for gliomas, we investigated the gene expression levels of cathepsin D in 87 tissue samples and measured the protein concentrations in sera of glioma patients. The tissue samples consisted of 43 glioblastomas, 13 anaplastic astrocytomas, 22 astrocytomas, and 9 normal brain tissues. The results of real-time quantitative reverse transcription-PCR analysis showed that cathepsin D transcript levels became significantly higher as the glioma grade advanced ($P = 0.0466$, glioblastoma and anaplastic astrocytoma; $P = 0.0008$, glioblastoma and astrocytoma; $P = 0.0271$, glioblastoma and normal brain tissue; unpaired $t$ test). Immunohistochemical analysis with anti-cathepsin D antibody revealed dense and spotty staining in the tumor cells with high transcript levels. The low expression of cathepsin D significantly correlated with long survival of the glioma patients. Furthermore, the glioblastoma patients with high gene expression of cathepsin D lived significantly shorter than those with low expression ($P = 0.0104$, Cox–Mantel log-rank test) and frequently had leptomeningeal dissemination ($P = 0.0016$, $\chi^2$ test). The multivariate analysis confirmed that the cathepsin D expression level was an independent predictor for short survival ($P = 0.0102$, Cox proportional hazard regression model). Measurement of the serum cathepsin D concentrations by ELISA showed a significant increase in the patients with high-grade gliomas as compared with the low-grade tumors ($P = 0.0081$, $\chi^2$ test). These results collectively suggest that cathepsin D could be a potential serum marker for the prediction of aggressive nature of human gliomas. (Cancer Res 2005; 65(12): 5190-4)

Introduction

Tumors of glial origin, such as glioblastoma, constitute the majority of primary brain tumors. The patients with glioblastoma have an average life expectancy of less than a year (1). The molecular pathogenesis of glioma includes both gain and loss of proteins responsible for the proliferation signals. Identification of the sets of genes that are differentially expressed between the high-grade gliomas and the low-grade tumors or normal brain tissues is important to understand the molecular basis of these nervous system tumors, to accurately predict the patient prognosis, and to develop novel therapeutic strategies (2). If the gene products can be detected in the patient sera, the clinical significance would be high for the preoperative diagnosis and for monitoring the response to therapy. With such a set of serum markers, patients would be better informed about the likely benefits of aggressive treatments.

The most devastating and therapeutically intractable aspect of glioblastoma is the disease highly invasive nature that prevents complete tumor resection and causes significant neurologic morbidity and mortality (1). The transformed cells release increased levels of proteolytic enzymes that facilitate tumor invasion (3, 4). Among the proteases, cathepsin D is an aspartyl protease that is normally localized within the lysosomes and involved in protein degradation and processing of the precursor proteins (5, 6). For example, it is responsible for specific cleavage and processing of myelin and other brain-associated proteins, conversion of procollagen into collagen, and activation of the inhibitors of cysteine proteases (3–6). It has also been suggested that this enzyme may be involved in several actions that facilitate the tumor progression, such as degradation of the extracellular matrix to promote tumor invasion (7–10). Elevated expression and secretion of cathepsin D have been noticed not only among the patients with breast cancer but also among those with other solid tumors (11), and it frequently correlates with poor prognosis (12, 13).

In the previous studies, we did differential proteomics associated with malignant transformation of human gliomas using the two-dimensional gel electrophoresis and mass spectrometry (2). We could identify the scores of proteins, which are abundantly expressed in the high-grade gliomas and can potentially predict the malignant nature of gliomas as serum markers. Especially, the high expression of cathepsin D is one of the common features in the tissue samples of the high-grade astrocytomas (2). Because cathepsin D has been reportedly secreted from cancer cells, we focused on this molecule as a potential serum marker for the diagnosis of glioma malignancy. We measured the degree of gene expression of cathepsin D in cDNA samples obtained from the glioma tissues, and also measured the serum concentration of cathepsin D in the glioma patients. We analyzed the correlation between the molecular information and the clinical data including the tumor grade, invasive nature, and survival period.

Materials and Methods

Tissue specimens. Seventy-eight gliomas (22 diffuse astrocytomas, 13 anaplastic astrocytomas, 43 glioblastomas) and nine normal brain tissues were examined. All tissue samples were obtained from the patients at the Chiba University Hospital under the protocol approved by the institutional review board, and informed consents were obtained from the patients or their guardians. The histopathologic diagnoses of all specimens were confirmed by two neuropathologists according to the criteria established...
by the WHO. All of the gliomas investigated were obtained at the time of each patient's first surgery. The normal samples were obtained from the overlying cortex during resection of deeply seated benign tumors. A portion of each sample was fixed in 10% formaldehyde and embedded in paraffin and the remaining sample was immediately frozen in liquid nitrogen.

**Extraction of mRNA and preparation of cDNA.** The mRNAs were extracted from the tumors and normal brain tissues using QIAzol Lysis Reagent and RNase Free Lipid Tissue Mini Kit (Qiagen, Tokyo, Japan) according to the instructions of the manufacturer. The electrophoretic purity of all the mRNA samples was confirmed. One microgram of each mRNA was reversely transcribed using the oligo dT primer (TAKARA BIO, Inc., Tokyo, Japan) and Super Script II (Invitrogen Corp., Carlsbad, CA) according to the method described by Yoshikawa et al. (14).

**Real-time reverse transcription-PCR.** Real-time quantitative reverse transcription-PCR (RT-PCR) was done using the Light Cycler (Roche Diagnostics, Meylan, France), which exploited the ability of SYBR green to fluoresce after hybridization with a double-strand DNA. The amplification was done using 5'-TATTGTGCAGGCACGGC-3' (Qiagen) as the forward primer and 5'-GACACTTGGACCCTAGCCCT-3' (Qiagen) as the reverse primer. The analyses were done in 20 μL glass capillaries using the Light Cycler fast start DNA master SYBR green kit (Roche Diagnostics). Then, 1 mmol/L of each primer and 3 mmol/L of MgCl2 in the total volume of 20 μL were used in each real-time RT-PCR amplification. The real-time RT-PCR cycle started with the initial denaturation at 95°C for 10 minutes, followed by 45 cycles of denaturation at 95°C for 10 seconds, annealing at 61°C for 10 seconds, and elongation 72°C for 10 seconds. As an internal quantitative control of the gene expression, the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene expression was determined as reported previously (15). The cathepsin D and GAPDH gene expressions of all cDNA samples were determined by fluorescence from SYBR green using the Light Cycler software Version 3.5 (Roche Diagnostics), and the ratios of cathepsin D and GAPDH gene expressions represented the normalized relative levels of cathepsin D expressions.

**Immunohistochemical analysis.** For immunohistochemical analyses of gliomas, paraffin-embedded samples were sliced and mounted on microscopic slides. Rabbit polyclonal anti Cathepsin D antibody 1:200 dilution, Santa Cruz Biotechnology, Santa Cruz, CA) was used as the primary antibody. Heat-induced epitope was formed with microwave in 0.01 mol/L citric acid buffer at pH 7.2. The samples were incubated with the antibody overnight in the same buffer followed by incubation with the biotinylated secondary antibody (1:500 dilution, DAKO, Tokyo, Japan). The bound antibodies were visualized by the avidin biotinylated peroxidase complex methods and diaminobenzidine tetrahydrochloride (Santa Cruz Biotechnology).

**ELISA of patient sera.** We collected serum samples from 20 patients diagnosed with various-grade gliomas. They were 12 preoperative patients, and 8 postoperative and preirradiation patients with apparent tumor on magnetic resonance imaging. Of the 20 patients who underwent ELISA, 6 were included among the original 87 patients. None of them was receiving steroid therapy at the time of blood sampling. All of the blood samples were allowed to clot at 4°C for no more than 3 hours, and were then centrifuged for 5 minutes at 1,000 rpm. The serum (upper phase) was aliquoted and stored at −80°C until use. ELISA plates (96-well) were coated with 20 μg/mL antihuman cathepsin D antibody (GT, Minneapolis, MN), and were filled overnight with 50 μL of patients' sera diluted 1:25. The plates were exposed to biotinylated antihuman cathepsin D antibody diluted at 1:500, and then to peroxidase-conjugated avidin at 1:1,000. The plates were developed with 3,3 diaminobenzidine (Sigma-Aldrich, St. Louis, MO) and were read at absorbance of 490 nm.

**Statistical analysis.** The survival periods of the patients with gliomas were calculated and the date of the initial surgery was set as zero. The Kaplan-Meier method was used to estimate the survival rates, and the Cox-Mantel log-rank test was applied to compare the survival differences among the patients using StatView software (SAS Institute, Inc., Cary, NC). We analyzed the correlation between the classification of cathepsin D expression into higher ratio or lower ratio and the patient's survival period. The other potential prognostic variables were age, sex, extent of surgery, and perioperative Karnofsky performance status score. Magnetic resonance images of all patients were obtained with and without Gd enhancement to assess the infiltrative or disseminated areas during their survival. After the surgery, the patients were treated with the conventional radiotherapy and chemotherapy. The multivariate analysis was done with the commercially available software by using the Cox proportional hazard regression model (SPSS, Inc., Chicago, IL).

**Results**

**Differential expression of the cathepsin D gene in astrocytic tumors.** The relative cathepsin D and GAPDH gene expression levels in the normal brain tissues, diffuse astrocytomas, anaplastic astrocytomas, and glioblastomas are shown in Fig. 1. The expression level was lowest in the normal brain tissue (0.036 ± 0.16, n = 9), and it increased as the histologic grading advanced. The value was 0.041 ± 0.02 for diffuse astrocytomas (n = 22), 0.139 ± 0.149 for anaplastic astrocytomas (n = 13), and 0.448 ± 0.538 for glioblastomas (n = 43). The unpaired t test indicated significant differences between diffuse astrocytoma and anaplastic astrocytoma (P = 0.0047), and between anaplastic astrocytoma and glioblastoma (P = 0.0466). There was no significant difference between the normal brain tissues and diffuse astrocytomas (P = 0.5356). These observations strongly suggest that the degree of relative cathepsin D gene expressions positively correlated with the progress of histologic grade in astrocytic tumors.

**Immunohistochemical staining for cathepsin D.** In addition to assessing the gene expression of cathepsin D, the level of gene product was assessed immunohistochemically. Although weak and reticular staining for antihuman cathepsin D antibody was observed in all grades of astrocytomas and some neurons in the normal brain, dense and spotty staining was observed only in the tumors with high cathepsin D expression ratios (Fig. 2). In contrast, glioblastomas with low cathepsin D expression ratios had large tumor areas never stained with the antibody. This result suggests that the cathepsin D expression level measured with real-time RT-PCR directly correlated with the protein production in the glioma tissues.

![Image 1. Real-time RT-PCR analysis for the expression of cathepsin D in various grades of astrocytic tumors and normal brain tissues. A, AA, and GB indicate diffuse astrocytoma, anaplastic astrocytoma, and glioblastoma, respectively. There are statistically significant differences between anaplastic astrocytoma and glioblastoma (P = 0.0466), diffuse astrocytoma and glioblastoma (P = 0.0008), diffuse astrocytoma and anaplastic astrocytoma (P = 0.0047), and normal brain tissue and glioblastoma (P = 0.027). Bars, mean; columns, SD.](www.aacrjournals.org)
Correlation between cathepsin D gene expression and survival period. All glioma samples were analyzed for the correlation of cathepsin D/GAPDH ratio with patients survival (observation) periods. It indicated that all the tumors from patients with long survival (>2 years) had low levels of cathepsin D/GAPDH ratio. In contrast, the tumors from patients with shorter survival (<2 years) had a wide range of cathepsin D/GAPDH ratio (Fig. 3A).

Because the highest cathepsin D/GAPDH value in anaplastic astrocytomas was 0.465, we compared the overall survival periods of patients with glioblastoma between the two groups [i.e., glioblastomas with cathepsin D/GAPDH expression ratios <0.465 (26 patients) and the tumors with ratios ≥0.465 (17 patients)]. The patients of the former group lived significantly longer than those of the other group (Fig. 3B, \( P = 0.0104 \), Cox-Mantel log-rank test). There was no significant difference between the two groups in the potential prognostic factors such as age, performance status, extent of surgical resection, and dose of radiotherapy. To confirm the prognostic value of the cathepsin D expression level, we did multivariate analysis for survival on the 43 glioblastoma patients (Table 1). We found that the cathepsin D expression level was an independent factor for poor prognosis (\( P = 0.0102 \)). The age was the only other significant independent predictor for overall survival (\( P = 0.0472 \)).

Correlation between cathepsin D expression and leptome-ningeal dissemination. To explore the biological mechanisms by which the high expression level of cathepsin D could affect the survival times of the patients with glioblastoma, the infiltrative areas of glioblastomas were examined with magnetic resonance imaging. Because cathepsin D takes part in degradation of the extracellular matrix and is strongly expressed by invading glioblastoma cells at the infiltrative margin (16), cathepsin D may be associated with the invasive nature of glioblastomas. Among the six patients with very high expression level of cathepsin D (\( ≥1.0 \)), three manifested leptomeningeal disseminations on the Gd-enhanced magnetic resonance imaging. The frequency (50%) was significantly higher than in the other 37 patients (cathepsin D expression level <1.0) in whom only 2 tumors (5.4%) manifested magnetic resonance imaging features suggestive of leptomeningeal dissemination (Fig. 4, \( \chi^2 \) test, \( P = 0.0016 \)).

Figure 2. Immunohistochemical analysis for antihuman cathepsin D antibody. Paraffin-embedded sections of representative glioblastomas were stained with the antibody against human cathepsin D. The photographs on the top row (A-C) are glioblastomas that have high levels of cathepsin D gene expression with dense and spotty staining for cathepsin D. In contrast, glioblastomas in the bottom row, which had low levels of cathepsin D expression ratio, showed only weak and reticular staining for the cathepsin D antibody (D) or large tumor areas that were not stained with the antibody (E and F).

Figure 3. A, scatter plot of the relation between survival period and cathepsin D/GAPDH ratio. All the tumors from the patients with long survival (>2 years) had low levels of cathepsin D/GAPDH ratio. In contrast, the gliomas from short-survived patients (<2 years) had a wide range of cathepsin D/GAPDH ratio. Among the patients with long survival (>2 years), several cases were still alive and their observation periods were used.

B, Kaplan-Meier survival curves of the patients with glioblastoma as divided by the real-time RT-PCR expression levels of cathepsin D. The patients with a gene expression level ≥0.465 (the highest value in anaplastic astrocytomas) and those with low expression tumors (<0.465) are compared. The difference between the two survival curves was statistically significant (log-rank test, \( P = 0.0104 \)).
Cathepsin \( D \) concentrations in the patient sera. On the basis of the aforementioned data, cathepsin \( D \) seems to be a promising candidate serum marker for diagnosis of the biological aggressiveness in gliomas. To test whether cathepsin \( D \) can be used as a serum marker correlating with the presence and grade of glioma, we measured the serum levels of cathepsin \( D \) in 20 patients with gliomas by ELISA (Fig. 5). Comparison of the serum cathepsin \( D \) levels was made between 9 patients with low-grade gliomas and 11 patients with high-grade gliomas. The mean values \( \pm SD \) were 16.2 \( \pm \) 32.3 ng/mL for the low-grade tumors and 242.0 \( \pm \) 329.1 ng/mL for the high-grade tumors. When a cutoff was set at 100 ng/mL, the positive ratio was significantly higher in the high-grade tumors than in the low-grade tumors (\( \chi^2 \) test, \( P = 0.0081 \)). Although the tumor tissues from the 20 patients were not necessarily available for analysis of the gene expression, all tumors from the patients with high serum concentrations expressed high cathepsin \( D \) transcripts.

Discussion

In this study, we showed that the transcript level of cathepsin \( D \) positively correlated with the histologic grade in the astrocytic tumors, and it could be used as a predictive marker for poor prognosis in the glioma patients. Furthermore, the serum levels of cathepsin \( D \) protein could be detected in the glioma patients, indicating that cathepsin \( D \) was secreted from a subset of gliomas. The serum protein level was significantly higher in the patients with high-grade gliomas as compared with the low-grade gliomas. These data suggest that the serum cathepsin \( D \) level may be a potential indicator of the biological aggressiveness of intracerebral gliomas. With its disease specificity and response to treatment demonstrated in further analysis, cathepsin \( D \) can be considered as a serum marker of tumor burden and recurrence in human gliomas.

Cathepsin \( D \) is an aspartic endopeptidase which is normally involved in the intralysosomal and intraendosomal cellular degradation of proteins and in the processing of precursor proteins (3–5). The coding gene is located on chromosome 11p15, and is a housekeeping gene ubiquitously present in all mammalian cells. Some authors have reported that overexpression of cathepsin \( D \) in the carcinoma cells of the breast and some other organs is associated with a higher risk of relapse and metastasis, and consequently with a shortened survival period (11, 12). For brain tumors, there are a few reports indicating the correlation between cathepsin \( D \) expression and the biological features of the tumor cells (16–18). Immunochemical studies of tissue extracts from glioblastomas showed increased levels of cathepsin \( D \) as compared with the other grade gliomas and normal brain tissues. In a recent study, cathepsin \( D \) antibody was shown to inhibit the invasion of a glioblastoma cell line (U251) in a dose-dependent manner (17). In an experimental mouse model of the human glioblastoma, an association between the cathepsin \( D \) immunoreactivity and the aggressive proliferative activity of U87 cells was noticed as compared with the less aggressive U373 cell line (18). These data are in accordance with our result.

Although the mechanisms by which overexpressed cathepsin \( D \) contributes to tumor relapse and metastasis including leptomeningeal dissemination are not fully understood, three pathways could be involved in this process. First, it has been suggested that cathepsin \( D \) acts as an autocrine mitogen by increasing the cell growth and decreasing the contact inhibition (7–10). Cathepsin \( D \) can be secreted from the tumor cells as a proenzyme (6, 8). At a neutral pH, the secreted pro-cathepsin \( D \) interacts with different

Table 1. Multivariate analysis by the Cox proportional hazard regression model in 43 glioblastoma patients

<table>
<thead>
<tr>
<th>Variable</th>
<th>( P )</th>
</tr>
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<tbody>
<tr>
<td>Age</td>
<td>0.0472</td>
</tr>
<tr>
<td>Sex</td>
<td>0.6901 (ns)*</td>
</tr>
<tr>
<td>KPS† score</td>
<td>0.3255 (ns)</td>
</tr>
<tr>
<td>Extent of surgery</td>
<td>0.0706 (ns)</td>
</tr>
<tr>
<td>Expression level of cathepsin ( D )</td>
<td>0.0102</td>
</tr>
</tbody>
</table>

*ns, not significant.
† KPS, Karnofsky performance status.

Figure 4. Correlation between very high expression of cathepsin \( D \) (cathepsin \( D/GAPDH \) ratio \( \geq 1.0 \)) and leptomeningeal dissemination in 43 glioblastoma patients. The Gd-enhanced magnetic resonance images show three glioblastoma cases with leptomeningeal dissemination. Black arrows, disseminated lesions. Among the six patients with very high levels of cathepsin \( D \) (\( \geq 1.0 \)), three patients (50%) manifested leptomeningeal dissemination.

<table>
<thead>
<tr>
<th>Leptomeningeal dissemination</th>
<th>Cathepsin ( D )</th>
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<tr>
<td>( \geq 1.0 )</td>
<td>( &lt; 1.0 )</td>
</tr>
<tr>
<td>−</td>
<td>3 (50%)</td>
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<tr>
<td>+</td>
<td>3 (50%)</td>
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* Diagnosed by gadolinium-enhanced MRI
† The normalized ratio of cathepsin \( D/GAPDH \)
‡ Percentage with raw numbers in parenthesis

\( \chi^2 \) test; \( p = 0.0010 \)
extracellular matrix and thereby facilitate tumor invasion and metastasis (20). The secreted pro-cathepsin D requires activation at a low pH to show its proteolytic activity (6, 11, 21). Extracellular pH in the tumor tissues is known to be more acidic than that in the normal tissues. Third, intracellularly, it may either activate the growth factor pathways or inactivate the growth inhibitors. Indeed, cathepsin D is reported to participate in a potent proteolytic cascade to activate pro-cathepsin B, cathepsin B, and the pro-urokinase plasminogen activator (3–6, 22, 23). Cathepsin B has been reported to be functionally important in the process of tumor invasion and angiogenesis during the malignant progression of gliomas (22, 24).

In conclusion, we have shown that measurement of the tissue cathepsin D expression levels can identify a subgroup of gliomas with highly aggressive nature and a high likelihood of leptomeningeal dissemination. Furthermore, this investigation is the first clinical study to show that the serum cathepsin D level correlated with the histologic grade of gliomas, suggesting that the serum cathepsin D level could be a potential indicator for disease aggressiveness in human gliomas.

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References

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