

High Serum Level of Plasminogen Activator Inhibitor-1 Predicts Histological Grade of Intracerebral Gliomas

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Abstract. *Background:* No serum marker is currently available for the diagnosis and treatment of gliomas. Plasminogen activator inhibitor-1 (PAI-1) controls the proteolytic activity in cancer cells and cellular migration during angiogenesis. *Patients and Methods:* To verify the potential of PAI-1 as a serum marker for gliomas, the serum PAI-1 concentrations were measured by ELISA in 57 glioma patients and 34 healthy volunteers. *Results:* We found significantly higher serum levels in the patients with high-grade gliomas than in the healthy volunteers ($p=0.0009$, unpaired t-test) and those with low-grade tumors ($p=0.0074$). Furthermore, high-grade glioma patients with a low serum level of PAI-1 survived significantly longer than those with high levels ($p=0.0082$). Immunohistochemical analysis using anti-PAI-1 antibody revealed dense and spotty staining in the high-grade tumor tissues from the patients with high serum PAI-1 levels. *Conclusion:* These results suggest that the serum PAI-1 level can be a marker for the prediction of histological grade in intracerebral glioma.

Gliomas are the most common primary brain tumors with a highly invasive nature that prevents complete tumor resection and causes significant neurological morbidity and mortality (1). The prognosis of patients with gliomas remains heterogeneous even among lesions with identical pathological diagnosis and histological grading. This is partly because the classification of this tumor roughly depends on histological grading alone. If the biological signatures which significantly correlate with the patients' prognosis can be detected in the peripheral blood, the clinical importance would be high for preoperative

prediction of the prognosis, treatment selection and serial monitoring of the response to therapy (2). With utilization of such biomarkers in panels, patients would be better treated according to the individual tumor's biological background and would be better informed about the likely benefits of proposed treatments.

In previous studies, we demonstrated by proteomics based on two-dimensional gel electrophoresis and mass spectrometry that a high expression of plasminogen activator inhibitor 1 (PAI-1) was one of the common features in the tissue samples of high-grade astrocytomas (3). PAI-1 is an important mediator of coagulation/fibrinolysis through inhibition of urokinase-type plasminogen activator (uPA) and tissue-type PA (tPA). In relation to cancer, PAI-1 is known to play a key role in extracellular matrix degradation and in the subsequent release of growth and angiogenic factors (4-6). Some authors have shown high levels of PAI-1 in the human tissue samples of various cancers and malignant gliomas (7-13). Although PAI-1 has been reportedly secreted from cancer cells and can be detectable in the patients' peripheral blood (13-15), only few data are available concerning the diagnostic value of the serum PAI-1 level in human gliomas.

Since, under normal conditions, PAI-1 is mainly produced from platelets and endothelial cells, it is usually measured in plasma not in serum. However, when used in a panel with other multiple markers to increase the reliability of cancer diagnosis, the measurement in serum is advantageous over the measurement in plasma. We focused on this molecule as a potential serum marker for the prediction of histological grade, and measured its serum concentrations in the glioma patients to elucidate its diagnostic value as a glioma marker.

Patients and Methods

Patients. Fifty-seven patients with gliomas (14 diffuse astrocytomas, 5 anaplastic astrocytomas, 26 glioblastomas, and 12 oligodendro-gliomas) were examined at Chiba University Hospital under the protocol approved by the institutional review board, and informed consents were obtained from the patients or

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their guardians. The histopathological diagnoses of all specimens were confirmed by two neuropathologists according to the criteria established by the World Health Organization (WHO) (1). All of the glioma tissues investigated were obtained at the time of each patient's first surgery.

In 7 of the 31 patients with high-grade gliomas, the PAI-1 concentrations were measured in matched-pair samples obtained from each of these patients at diagnosis and after completion of therapy with good tumor control. In order to assess possible differences in serum PAI-1 concentrations between healthy individuals and glioma patients, peripheral blood samples from 34 healthy volunteers were also examined. They were free from any type of cancer or infectious disease.

ELISA of the sera. Blood samples were taken from the patients with different grade gliomas and from the healthy volunteers. Patients harbored apparent neoplastic lesions on MRI. None of them was receiving steroid therapy at the time of blood sampling. Immediately after removal, venous blood samples were centrifuged at room temperature for 5 min and stored at -80°C until analysis. Quantification of PAI-1 was performed using a commercially-available kit (Human PAI-1 Activity Assay, Innovation Research, Inc., Greenwich, CT, USA). ELISA plates (96-well) were coated with uPA, and then filled with 20 µl of the patients' serum diluted with general assay diluent. The wells were exposed to monoclonal anti-PAI-1 antibody and then to horseradish peroxidase-labeled secondary antibody. The plates were developed with 3,3',5,5'- tetramethylbenzidine (TMB) substrate solution. The reaction was quenched with 1N H₂SO₄ and read at an optical density (OD) of 450 nm. The PAI-1 concentration was determined from a standard curve plotting the absorbance at 450 nm (A_{450}) against the amount of PAI-1 in the standards.

Immunohistochemical analysis. For immunohistochemical analysis, paraffin-embedded glioma samples were sliced and mounted on glass slides. Rabbit monoclonal PAI-1 antibody (1:200 dilution; Santa Cruz Biotechnology, Santa Cruz, CA, USA) was used as the primary antibody. Heat-induced epitope was formed in 10 mM citric acid buffer at pH 7.2 using microwaves. 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 using the avidin biotinylated peroxidase complex methods and diaminobenzidine tetrachloride (Santa Cruz Biotechnology).

Statistics. The survival periods of the patients were calculated by setting the date of the initial surgery 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, USA).

Results

Serum PAI-1 level in glioma patients. On the basis of the aforementioned data, PAI-1 appears to be a promising candidate biomarker for diagnosis of the biological aggressiveness of gliomas. To test whether PAI-1 can be

Table I. Clinical backgrounds of glioma patients and healthy volunteers.

	High-grade gliomas	Low-grade gliomas	Healthy volunteers
Number of cases (n)	31	26	34
Gender			
Male	21	15	20
Female	10	11	14
Mean age (years; range)	55 (18-79)	43 (16-64)	54 (23-67)
Serum PAI-1 level (n)			
<10 ng	15	26	34
≥10 ng	16	0	0
Immunohistochemistry			
Positive	13	2	-
Negative	7	18	-

used as a serum marker *via* its association with the presence and grade of glioma, we compared the serum PAI-1 levels between 26 patients with low-grade gliomas and 31 patients with high-grade gliomas (Table I). These patients were confirmed to harbor neoplastic lesions on MRI at the time of blood sampling. The mean values±SD of the serum PAI-1 concentration were 3.54 ± 9.27 ng/ml for the low-grade tumors and 14.7 ± 20.2 ng/ml for the high-grade tumors (unpaired *t*-test, $p=0.0074$) (Figure 1). When a cut-off was set at 10.0 ng/ml, the positive ratio was also significantly higher in the high-grade tumors than in the low-grade tumors (χ^2 test, $p=0.0001$). The mean serum concentration of 7 cases in whom high-grade gliomas were successfully treated without recurrence was 4.45 ± 9.50 ng/ml. Although serum PAI-1 level decreased after treatment in high-grade glioma patients, the difference from their pre-treatment values did not reach statistical significance (Figure 1).

Correlation between serum PAI-1 level and survival in high-grade gliomas. Since all the serum PAI-1 levels in low-grade gliomas were under 10.0 ng/ml, we compared the overall survival periods of high-grade gliomas between the high serum PAI-1 group (serum PAI-1 ≥ 10.0 ng/ml; 16 cases) and the low serum PAI-1 group (serum PAI-1 < 10.0 ng/ml; 15 cases). The low serum PAI-1 group survived significantly longer than the high serum PAI-1 group (Figure 2, $p=0.0082$, log-rank test). There was no significant difference between the two groups as regards the potential prognostic factors.

Immunohistochemistry for tissue PAI-1 expression. Surgically-resected tumor tissues were examined by immunohisto-chemistry using anti-PAI antibody. The immunohisto-chemical positive rate was significantly higher in the high-grade gliomas than in low-grade

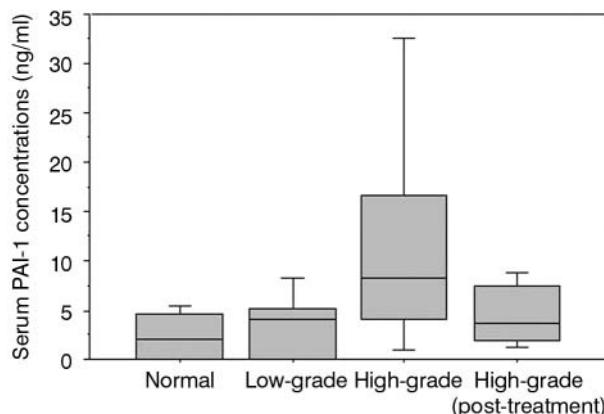


Figure 1. Boxplots of serum PAI-1 concentrations in healthy volunteers and glioma patients; low-grade gliomas, high-grade gliomas, and successfully treated high-grade gliomas without tumor progression. There are significant differences in the serum PAI-1 levels between the patients with low-grade and those with high-grade gliomas ($p=0.0074$, unpaired t -test). Vertical lines indicate the smallest and the largest non-outlier observations, and boxes indicate the lower and the upper quartiles.

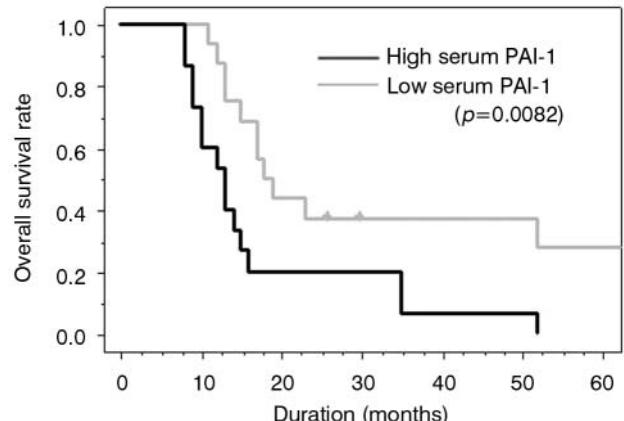


Figure 2. Kaplan-Meier survival curves of the patients with high-grade gliomas as divided by the serum PAI-1 levels. The patients with a serum PAI-1 level of ≥ 10 ng/ml and those with low levels < 10 ng/ml are compared. The difference between the two survival curves was statistically significant (log-rank test, $p=0.0082$).

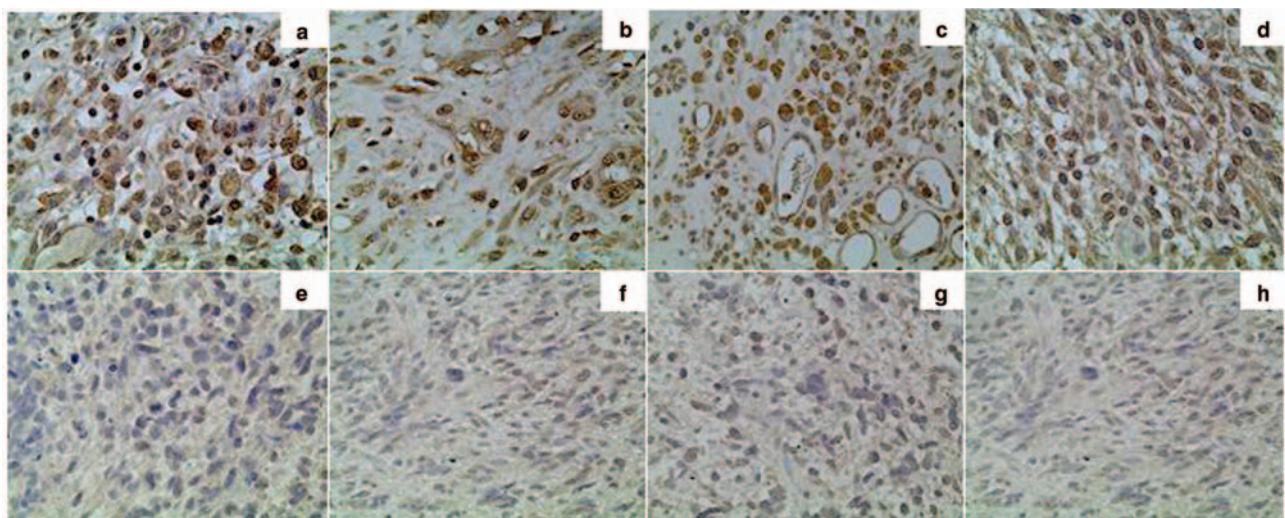


Figure 3. Immunohistochemical analysis of the anti-human PAI-1 antibody. The tumors from the patients with a high serum PAI-1 level showed dense and spotty staining of PAI-1 (a, b, c). In contrast, the tumors that had low serum levels of PAI-1 (d, e, f) showed only weak and reticular staining of the PAI-1 antibody or had large tumor areas that were not stained.

gliomas (χ^2 test, $p=0.0003$) (Table I). A dense and spotty immunostaining in the cytoplasm was observed only in the high-grade gliomas with high serum PAI-1 levels (Figure 3). In contrast, weak and reticular staining of the anti-human PAI-1 antibody was occasionally observed in all grades of astrocytomas and in some neurons in the surrounding normal brain tissues.

Discussion

Although PAI-1 is usually measured in plasma not in serum as a marker of coagulation/fibrinolysis activity, we successfully measured serum PAI-1 levels and showed that the serum PAI-1 level was significantly higher in the patients with high-grade gliomas than in those with the low-grade

tumors or in the healthy volunteers. The elevated PAI-1 level could be reduced by successful treatments including surgery, radiotherapy and chemotherapy. The data suggest that PAI-1 is secreted from a subset of high-grade gliomas and that its serum level may be a potential biomarker for the biological aggressiveness of intracerebral gliomas. When combined with contemporary imaging studies such as Gd-enhanced MRI, the PAI-1 level in the peripheral blood can act as a functional marker of biologically-active neoplastic lesions or tumor recurrence. When used in a panel with other serum markers for cancer diagnosis, the measurement in serum is advantageous over plasma measurements.

PAI-1 is the primary inhibitor of uPA and tissue-type PA (tPA), and consequently inhibits the activation of plasminogen into plasmin. In cancer cells, uPA and tPA at the cell surface initiate a protease cascade which leads to breakdown of the extracellular matrix (5, 6). By blocking the interaction between vitronectin, uPAR and integrins, PAI-1 may induce the detachment of cancer cells from the extracellular matrix and promote cellular migration and tumor invasion (4-6). Although there are some controversies concerning the PAI-1 expression and tumor invasion in the experimental studies (16, 17), the high levels of uPA and PAI-1 in tissue or serum/plasma have been demonstrated in the clinical studies on patients having the most aggressive breast cancers, and thus they could be biomarkers for poor prognosis (7, 8).

Regarding brain tumors, some studies revealed a correlation between the tissue PAI-1 expression and the histological malignancy (9-12). Recently, it was reported that PAI-1 produced by glioma may also increase its peripheral levels (13). From these results and our previous proteome investigation, this molecule was expected to be a candidate biomarker for malignant gliomas. For gliomas, no secreted protein that is specific enough to be utilized as a biomarker has yet been identified. Since PAI-1 is not a glioma-specific protein, it cannot serve as a single diagnostic marker for mass screening. However, the search for such candidate biomarkers, although not glioma-specific but significantly up-regulated, would yield a novel diagnostic system when used in appropriate combinations.

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