Macrophage Infiltration-associated Thymidine Phosphorylase
Expression Correlates with Increased Microvessel Density
and Poor Prognosis in Astrocytic Tumors

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ABSTRACT

Purpose and Experimental Design: To clarify the significance of thymidine phosphorylase (TP)/platelet-derived endothelial cell growth factor/gliostatin in human glioma, we examined TP expression immunohistochemically in a series of 50 astrocytic tumors and correlated its expression with tumor angiogenesis and apoptosis, as well as prognosis.

Results: The majority of TP-positive cells were of macrophage origin, which was confirmed by immunostaining TP and CD68 on mirror sections. TP expression was significantly associated with glioma malignancy grading, intratumoral microvessel density, and VEGF expression but showed no relationship with apoptotic index or P53 expression. Regardless of glioma grading, patients with TP-positive tumors had a significantly shorter mean survival time than those with TP-negative tumors.

Conclusions: These findings suggest that TP might play a crucial role in angiogenesis during glioma development, and immunodetection of TP is useful for clinical prediction. Further studies are necessary to better elucidate the role of TP in glioma, which may provide insights into adequate TP-targeted therapy.

INTRODUCTION

TP2 is an intracellular enzyme involved in the reversible conversion of thymidine to thymine (1). Recent studies demonstrated that TP is completely identical to platelet-derived endothelial cell growth factor, a novel angiogenic factor (2–5). Compared with adjacent nonneoplastic tissues, higher TP expression is found in a wide variety of solid tumors, including human glioma (6, 7). TP stimulates chemotaxis and [3H]thymidine incorporation of endothelial cells in vitro and is strongly angiogenic in vivo (8–10). In addition, TP can confer resistance to hypoxia-induced apoptosis (11). The enzymatic activity of TP is indispensable for its each function (8, 9, 11–13).

The ability to identify early prognostic marker(s) may aid in the clinical management of tumor patient. We demonstrated previously that VEGF, another important angiogenic factor (14), has a significant prognostic value in astrocytic tumors (15). The unique bifunctional properties of TP in tumor biology suggest that it may be also a reliable prognostic marker. In fact, overexpression of TP has been correlated with poor prognosis in a range of tumor types, such as breast, lung, gastric, colorectal, bladder, and cervical cancers (16–21).

However, the significance of TP expression in glioma remains unclear because of the fact that TP is also known as gliostatin, an inhibitory growth regulator against all glial cells (22, 23). To clarify the role of TP in glioma development and its value as a prognostic factor, we evaluated TP expression immunohistochemically in a series of 50 astrocytic tumors with different malignancy and correlated its expression with tumor angiogenesis and apoptosis, as well as patient prognosis.

MATERIALS AND METHODS

Patients and Specimens. Tumor specimens were obtained from previously untreated astrocytic tumor patients who underwent surgical treatment at Fukui Medical University Hospital from 1987 to 1998. Tumors were excluded if they were located in hypothalamus, thalamus, or posterior fossa or if the amount of sample was insufficient for immunostaining. There were 15 LGAs, 8 AAs, and 27 GBM, diagnosed according to the WHO classification. The age of patients (35 males and 15 females) ranged from 17 to 83 years of age (average, 51.3 years). All patients received postoperative irradiation and chemotherapy. An additional 3 autopsied normal adult brains were also examined. Specimens were fixed with 10% buffered formalin and embedded in paraffin.

Immunohistochemistry. The staining procedure was achieved by the EnVision+ technique (DAKO) on 2-μm-thick consecutive sections. Primary antibodies raised against TP (IC6-203, Nippon Roche; 1:2000 dilution), CD31 (JC-70A, DAKO; 1:100 dilution), P53 (DO7, Novocastra; 1:200 dilution), VEGF (Ab-2, Calbiochem; 1:40 dilution), Ki-67 (DO7, Novocastra; 1:200 dilution), and GFAP (DAKO; 1:200 dilution) were used. For epitope retrieval, specimens were pretreated with microwave (400 W, 2 min) or 0.1% protease (37°C, 5 min, for CD31 and VEGF) before incubation with primary antibodies (room temperature, 60 min). Negative controls were parallel sections treated as above with the omission of primary antibodies.

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2 The abbreviations used are: TP, thymidine phosphorylase; VEGF, vascular endothelial growth factor; LG, low-grade astrocytoma; AA, anaplastic astrocytoma; GBM, glioblastoma(s) multiforme; ssDNA, single-stranded DNA; GFAP, glial fibrillary acidic protein; MVD, microvessel density; AI, apoptotic index; KI, Ki-67 labeling index; MST, mean survival time.

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primary antibodies. Positive staining was visualized with diaminobenzidine tetrahydrochloride, followed by light hematoxylin counter staining. To identify the histological type of TP-positive cells, a further immunostaining TP together with CD68, a macrophage marker, or with GFAP, a glial cell marker, on several mirror sections was performed.

Assessment of TP, CD68, P53, and VEGF Immunoreactivity. Immunoreactivity of TP, CD68, P53, and VEGF was assessed by both staining intensity and percentage of positive cells. Tumors were considered as positive for these factors only when >10% of cells demonstrated moderate and/or strong staining intensity.

Assessment of Angiogenesis, Apoptosis, and Proliferative Potential. Intratumoral microvessels were highlighted by immunostaining endothelial cells for CD31 antigen, and a MVD was counted on a ×200 field (about 0.15 mm²) to measure the degree of angiogenesis, as described previously (24).

Apoptotic cells were highlighted by ssDNA immunostaining, and a AI, which is the percentage of ssDNA-positive cells to the total number (≥2000) of cells in the most frequently labeled area, was calculated to assess the degree of apoptosis. A KI was calculated similarly to assess the proliferative potential of tumors.

Statistical Analysis. Relationships between TP expression and glioma grading, MVD, AI, KI, P53, VEGF, and CD68 were examined by χ² test or Student’s t test. Survival curves were calculated by the Kaplan-Meier method and analyzed with the log-rank test. Statistical significance was defined as P < 0.05.

Table 1  Correlation between TP expression and glioma grading

The frequency of TP positivity significantly increased with glioma malignancy (χ² test).

<table>
<thead>
<tr>
<th></th>
<th>LGA (n = 15)</th>
<th>AA (n = 8)</th>
<th>GBM (n = 27)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>TP positive</td>
<td>3 (20%)</td>
<td>3 (37.5%)</td>
<td>19 (70.4%)</td>
<td>0.0056</td>
</tr>
<tr>
<td>TP negative</td>
<td>12 (80%)</td>
<td>5 (62.5%)</td>
<td>8 (29.6%)</td>
<td></td>
</tr>
</tbody>
</table>

Fig. 1  Representative results of immunostaining TP (A), CD68 (B), CD31 (C), ssDNA (D), VEGF (E), and P53 (F). A–F were sections from the same GBM. A and B were mirror sections. Notice that most TP-positive cells were also CD68 positive. Microvessels and apoptotic cells were clearly highlighted by CD31 and ssDNA immunostaining, respectively. Scale bars, 25 μm.
Forty-six (92%) of the 50 tumor specimens demonstrated TP immunoreactivity, and 25 (50%) cases were categorized as TP positive, whereas no TP immunoreactivity could be detected in the three normal brains. The frequency of TP positivity significantly increased with glioma malignancy ($P = 0.0056$; Table 1). GBM had a significantly higher frequency of TP expression than LGA ($P = 0.0017$, $\chi^2$ test).

### RESULTS

**Localization of TP Immunoreactivity.** Macrophage-like cells showed cytoplasmic TP immunoreactivity, which were often found within or around tumor vasculature and/or necroses. Immunostaining TP with CD68 or GFAP on several mirror sections demonstrated that most TP-positive cells were also positive to CD68 (Fig. 1, A and B) but were negative to GFAP (data not shown), which confirmed that the majority of TP-positive cells were of macrophage origin.

**Correlation between TP Expression and Glioma Grading.** Forty-six (92%) of the 50 tumor specimens demonstrated TP immunoreactivity, and 25 (50%) cases were categorized as TP positive, whereas no TP immunoreactivity could be detected in the three normal brains. The frequency of TP positivity significantly increased with glioma malignancy ($P = 0.0056$; Table 1). GBM had a significantly higher frequency of TP expression than LGA ($P = 0.0017$, $\chi^2$ test).

**Relationships between TP Expression and MVD, AI, KI, P53, VEGF, and CD68.** As shown in Table 2, regardless of glioma grading, TP-positive tumors had a higher mean MVD than those negative tumors, which was significant in LGA and GBM ($P = 0.0011$ and 0.0012, respectively). No significant relationship between TP and AI, KI, P53, or CD68 was found. However, we observed a close relationship between TP and VEGF, both in LGA and GBM ($P = 0.0062$ and 0.0011, respectively).

**Prognostic Significance of TP Expression.** We analyzed prognostic values of TP and other clinicopathological factors within each tumor grade. Regardless of glioma grading, patients with TP-positive tumors had significantly shorter MSTs than those with TP-negative tumors (log-rank test).

### Table 2

<table>
<thead>
<tr>
<th></th>
<th>MVD</th>
<th>AI</th>
<th>KI</th>
<th>P53 +</th>
<th>VEGF +</th>
<th>CD68 +</th>
</tr>
</thead>
<tbody>
<tr>
<td>LGA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TP +</td>
<td>31.4 ± 4.2$^a$</td>
<td>0.3 ± 0.1</td>
<td>2.2 ± 0.4</td>
<td>2 (66.7%)</td>
<td>3 (100%)</td>
<td>3 (100%)</td>
</tr>
<tr>
<td>TP −</td>
<td>16.5 ± 1.5</td>
<td>0.7 ± 0.2</td>
<td>1.7 ± 0.4</td>
<td>3 (25.0%)</td>
<td>2 (16.7%)</td>
<td>6 (50.0%)</td>
</tr>
<tr>
<td>AA</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TP +</td>
<td>33.0 ± 11.9</td>
<td>1.4 ± 0.8</td>
<td>6.5 ± 1.5</td>
<td>2 (66.7%)</td>
<td>2 (66.7%)</td>
<td>3 (100%)</td>
</tr>
<tr>
<td>TP −</td>
<td>20.5 ± 0.9</td>
<td>1.1 ± 0.5</td>
<td>6.5 ± 1.5</td>
<td>2 (40.0%)</td>
<td>3 (60.0%)</td>
<td>4 (80.0%)</td>
</tr>
<tr>
<td>GBM</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TP +</td>
<td>45.7 ± 4.2$^a$</td>
<td>0.5 ± 0.1</td>
<td>10.6 ± 1.4</td>
<td>12 (63.2%)</td>
<td>18 (94.7%)</td>
<td>19 (100%)</td>
</tr>
<tr>
<td>TP −</td>
<td>21.3 ± 2.2</td>
<td>0.7 ± 0.2</td>
<td>12.9 ± 2.1</td>
<td>3 (37.5%)</td>
<td>3 (37.5%)</td>
<td>5 (62.5%)</td>
</tr>
</tbody>
</table>

$^a$ Values are expressed as mean ± SE or as n (%).

$^b$ $P < 0.01$ versus control; +, positive; −, negative.

### Table 3

MSTs of patients with astrocytic tumors grouped according to various clinicohistopathological factors within each tumor grade.

<table>
<thead>
<tr>
<th></th>
<th>LGA</th>
<th>AA</th>
<th>GBM</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>MST ± SE</td>
<td>$P$</td>
<td>MST ± SE</td>
</tr>
<tr>
<td>Age</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥median</td>
<td>109.9 ± 15.4</td>
<td>NS$^a$</td>
<td>17.3 ± 2.3</td>
</tr>
<tr>
<td>&lt;median</td>
<td>98.4 ± 7.1</td>
<td></td>
<td>30.3 ± 7.9</td>
</tr>
<tr>
<td>MVD</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥median</td>
<td>85.8 ± 10.8</td>
<td>0.0152</td>
<td>17.3 ± 2.1</td>
</tr>
<tr>
<td>&lt;median</td>
<td>117.7 ± 10.2</td>
<td></td>
<td>36.0 ± 8.6</td>
</tr>
<tr>
<td>AI</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥median</td>
<td>104.9 ± 9.2</td>
<td>NS</td>
<td>25.3 ± 5.9</td>
</tr>
<tr>
<td>&lt;median</td>
<td>80.5 ± 4.5</td>
<td></td>
<td>14.0 ± 1.2</td>
</tr>
<tr>
<td>KI</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>≥median</td>
<td>90.3 ± 11.3</td>
<td>0.0438</td>
<td>29.3 ± 8.6</td>
</tr>
<tr>
<td>&lt;median</td>
<td>110.4 ± 5.8</td>
<td></td>
<td>18.3 ± 1.3</td>
</tr>
<tr>
<td>TP</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>73.5 ± 10.5</td>
<td>0.0067</td>
<td>15.3 ± 2.6</td>
</tr>
<tr>
<td>−</td>
<td>112.2 ± 8.4</td>
<td></td>
<td>33.4 ± 6.4</td>
</tr>
<tr>
<td>P53</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>91.5 ± 7.9</td>
<td>NS</td>
<td>18.3 ± 1.3</td>
</tr>
<tr>
<td>−</td>
<td>116.4 ± 11.6</td>
<td></td>
<td>29.3 ± 8.6</td>
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<tr>
<td>VEGF</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>76.0 ± 6.6</td>
<td>0.0005</td>
<td>16.8 ± 1.8</td>
</tr>
<tr>
<td>−</td>
<td>116.7 ± 8.3</td>
<td></td>
<td>35.3 ± 8.9</td>
</tr>
<tr>
<td>CD68</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>+</td>
<td>105.6 ± 11.4</td>
<td>NS</td>
<td>NA</td>
</tr>
<tr>
<td>−</td>
<td>90.0 ± 2.3</td>
<td></td>
<td>NA</td>
</tr>
</tbody>
</table>

$^a$ NS, not significant; NA, not applicable; +, positive; −, negative.
factors including patient age, MVD, AI, KI, P53, VEGF, and CD68 within LGA, AA, and GBM separately. Table 3 describes the MSTs of patients grouped according to various parameters within each tumor grade. Regardless of glioma grading, patients with TP-positive tumors had a significantly shorter MST than those with TP-negative tumors ($P = 0.0067$, $0.0499$, and $0.0082$ in LGA, AA, and GBM, respectively; Table 3; Fig. 2). MVD and VEGF were also significant prognostic factors, both in LGA and GBM. However, KI only demonstrated prognostic value in LGA. Other factors such as patient age, AI, P53, and CD68 did not show any prognostic significance.

**DISCUSSION**

In the present study, using a new monoclonal antibody specific to human thymidine phosphorylase (25), we found that the majority of TP-positive cells were of macrophage origin, which was confirmed by immunostaining TP and CD68 on mirror sections. Our results are consistent with those of Nakayama et al. (26), who used a double immunostaining method, but are different from those of Takano et al. (27), who reported that most TP-positive cells were tumor cells themselves. Interestingly, we found that not all macrophages necessarily expressed TP, as shown in Table 2. Some tumors, especially those of LGA, expressed very low or undetectable TP, despite their marked macrophage infiltration. Previous studies revealed that TP expression can be induced by inflammatory cytokines (28), and under hypoxia, low pH or ill-nourished microenvironment (29), which is usually present in solid tumors with high malignancy and/or rapid growth. We speculate that such mechanisms are also involved in glioma. Macrophages attracted and activated by above glioma-derived microenvironmental factors may, therefore, express TP and play some important roles in the development and progression of glioma.

Increased TP activity promotes angiogenesis in a range of pathologies (30). We observed a close relationship between TP expression and the degree of tumor angiogenesis, except for AA (perhaps because of the too small number of sample). The molecular mechanism underlying this link remains unclear because TP is not a classic secretory angiogenic factor (3). Brown et al. (31) recently demonstrated that TP can induce cell oxidative stress and promote secretion of other direct-acting angiogenic factors, such as VEGF and interleukin 8. Our study also showed that TP expression is significantly associated with VEGF expression, suggesting that TP may promote angiogenesis through triggering tumor cell secretion of VEGF, the central mediator in glioma angiogenesis (14). In fact, tumor angiogenesis is mediated by a number of angiogenic factors (32, 33), which may be involved in the vascularization of each tumor and...
function at different stages during tumor evolution (34). Further investigations should be performed to reveal the interrelationships between various angiogenic factors.

Recent studies indicated that TP is also involved in hypoxia-induced apoptosis (11, 13), although the precise mechanism has not been elucidated. TP expression has been correlated with decrease of apoptosis in colorectal, gastric, and ovarian carcinomas (35–37). However, our results showed no association between TP expression and apoptotic index determined by highlighting apoptotic cells with ssDNA immunostaining, a specific and sensitive detection method even better than the most widely used terminal deoxynucleotidyl transferase-mediated dUTP-digoxigenin nick end labeling method (38). This discrepancy might be explained that glioma is a special tumor, in which TP also acts as gliostatin, a selective growth inhibitor against glioma cells (22, 23). Therefore, the effect of TP conferring resistance to apoptosis may be abolished by its inhibitory effect on cell proliferation in glioma.

The prognosis of glioma patients is largely determined by the histopathological malignancy grade, although within each grade the clinical course of glioma patients is still variable because each grade of tumor is not a single pathological entity but encompasses a spectrum of tumors with variable malignant potential. The prediction of tumor biological behavior thus can be hardly made only by histological criteria and furthermore needs the predicting assistance of biological marker(s). Leon et al. (39) reported that vascular density is a diagnostic indicator for glioma patients. We and others revealed recently that VEGF has a significant prognostic value in astrocytic tumors (15, 40). Our present study demonstrated that TP is also a reliable prognostic marker, which has a negative impact on overall survival. However, this association between TP expression and poor prognosis does not necessarily mean that TP only facilitates glioma progression. It is also possible that TP is involved in the host defense mechanism against astrocytic tumors because of following reasons: (a) TP is expressed in a limited way in infiltrating macrophages, which usually play an important role in immune reactions; (b) TP is also known as gliostatin, which selectively inhibits astrocyte cells as well as astrocytoma cells but has a novel neurotrophic action on neurons (22, 23, 41); and (c) TP activity is a major determinant of the toxicity of the anticancer agent 5-fluorouracil and its prodrugs (42, 43). We infer that TP might be produced by infiltrating macrophages as a defense against tumor cells but simultaneously facilitates tumor growth, probably via promoting angiogenesis and preventing hypoxia-induced apoptosis. Alternatively, the apparent relation between TP expression and poor prognosis may be also explained that TP status is just a sensitive marker reflecting the microenvironmental conditions constructed by tumor cells, i.e., the biological behavior or malignancy of tumors.

In summary, our findings clearly indicated that macrophage infiltration-associated TP expression correlates with increased microvessel density, VEGF expression, and poor prognosis in astrocytic tumors. Immunodetection of TP expression on surgical specimens might be useful for clinical prediction of glioma patients. Additional studies are necessary to better elucidate the role of TP in glioma, which may provide insights into adequate TP-targeted therapy.

ACKNOWLEDGMENTS

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REFERENCES


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