Immunohistochemical Localization of Thymidine Phosphorylase in Gastric Cancer: Is there a Role of the Differential Expression in Tumor Cells and Associated Stromal Cells?

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Abstract. *Background: The aim of this study was to investigate the expression of thymidine phosphorylase (TP), a known angiogenic factor for endothelial cells, in gastric carcinoma cells and tumor-associated stromal cells. Materials and Methods: Sixty-six gastric carcinomas were studied. TP expression was assessed with the P-GF.44C mouse monoclonal antibody using the avidin-biotin immunoperoxidase technique. The results were correlated with several clinicopathological parameters and patient survival. Results: TP expression in cancer cells was related to the age of the patients and the overall survival. When TP was expressed in tumor-associated stromal cells, it was statistically related to poorly-differentiated tumors. Statistical analysis revealed no relationship between TP expression and any of the clinicopathological parameters under evaluation, when considering stromal TP immunoreactivity separately for stromal fibroblasts and associated inflammatory cells, or when considering the tumors as TP-positive, irrespective of the tissue localization of the enzyme. Conclusion: TP seems to be a prognostic indicator in gastric cancer patients only when the enzyme is located in tumor cells. The different impacts of TP expression in tumor cells and associated stromal cells might indicate that the enzyme may have more than one function involved in tumor growth.*

Thymidine phosphorylase (TP), also known as platelet-derived endothelial cell growth factor (PD-ECGF), is an enzyme specifically involved in the reversible dephosphorylation of thymidine to thymine and 2-deoxy-D-ribose-1-phosphate (1, 2). Several studies on human carcinoma tissues revealed that TP may be involved in tumor growth, mainly as an angiogenic factor having chemotactic activity on endothelial cells but also as a mitogenic factor which suppresses apoptosis (3-5).

The immunohistochemical expression of TP in several human solid tumors, including colorectal, breast, lung, prostate, urinary bladder and squamous esophageal, has been widely studied and has been shown to be correlated with metastasis and recurrence of the disease (6-11). In gastric cancer, most investigators found a correlation between TP-positive gastric cancers, clinicopathological parameters and postoperative survival (12, 13). Furthermore, TP positivity was considered as an independent prognostic factor indicating aggressive behavior of the tumor probably by promoting hematogenous and peritoneal metastasis (14). In most of these studies, mainly the immunohistochemical expression of the neoplastic cells was evaluated. Yet, it is known that TP is also expressed by tumor-associated stromal (TAS) cells, including fibroblasts, lymphocytes and macrophages. Since angiogenesis is induced, not only by angiogenic factors produced by tumor cells, but also by non-malignant cells embedded in the tumor (15), this retrospective study on gastric carcinomas was undertaken, in order to investigate the possible relationship of TP-positive cancer cells and tumor-associated stromal cells, including lymphocytes and macrophages, to several clinicopathological parameters and patient survival.

Materials and Methods

From 1999 to 2003, 66 gastric cancer cases were retrieved from the archives of the Pathology Departments of two affiliated hospitals. All

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samples were obtained with the consent of the patient or the bereaved and after approval of the local ethics committee. Forty-two patients were males (63.64%) and twenty-four were females (36.36%). The median age of the patients was 70 years (36-88 years). The patient follow-up was from 50-80 months or until death. The 5-year survival rate was 24.5%.

According to the 1998 revision of the TNM classification system by the joint meeting of the AJCC (American Joint Committee for Cancer) and the UICC (International Union Against Cancer), two (3.03%) early gastric cancers (Stage IA and IB), 18 (27.27%) Stage II, 39 (59.09%) Stage III (15 IIIA and 24 IIIB) and seven (10.61%) Stage IV tumors were encountered.

Histological grading was evaluated according to the Lauren classification system. There were 34 (51.52%) samples of intestinal type, 20 of diffuse (30.30%) and 12 (18.18%) of mixed type. Eleven samples (16.67%) were characterized as high grade, 14 (21.21%) as medium and 41 (62.12%) as low grade.

Immunohistochemistry. All the available hematoxylin and eosin-stained slides were reviewed and representative paraffin blocks from each case were selected for immunohistochemical study. One 4-μm thick section was cut from each block and placed onto a positively-charged glass-slide. The slides were then deparaffinized and endogenous peroxidase activity was blocked by incubation in 3% H_2O_2 in methanol for 30 min at room temperature. After washing in Tris-buffered saline (TBS), sections were incubated in normal human serum (1:10) for 20 min. The sections were then washed with TBS for 5 min and incubated with the P-GF,44C mouse monoclonal antibody (1:75) for 30 min. After washing with TBS for 5 min, the sections were incubated with biotynilated goat antimouse immunoglobulins (1:200) for 30 min (Dako, UK). After incubation with strept ABComplex/HRP (Dako) for 30 min, the peroxidase reaction was developed using diaminobenzidine as chromogen and the sections were counterstained with hematoxylin. For the assessment of tumor staining, normal mucosal-associated lymphoid tissue was used as an internal control. Omission of the primary antibody was used as a negative control.

Scoring system. The sections were examined using light microscopy by two independent observers (K.P. and I.G.) who were unaware of the clinicopathological data. Interobserver variation was resolved by simultaneous dual re-evaluation. Staining was evaluated over the entire tumor section.

Immunoreactivity for TP was evaluated with reference to both the staining intensity and the positively-stained area. Staining intensity was scored as follows: 0, none; 1, weak; 2, moderate; 3, strong. The positively stained area was expressed as the percentage of the whole cancer area and scored as: 0, none; 1, 0-25%; 2, 26-50%; and 3, >51%.The sum of scores less than or equal to 2 (≤ 2) was defined as negative (TP –) and more than or equal to 3 (≥ 3) as positive expression (TP+).

Statistical analysis. Categorical characteristics and their association with TP expression were assessed using the Pearson χ^2-test (16). Continuous characteristic comparisons were performed using the Mann-Whitney *U*-test. The probability of survival was calculated by the Kaplan-Meier method and differences in survival were assessed by the Log-rank and the Wilcoxon (Pento-Prentice) tests (17). All statistical tests were performed on a $p=0.05$ level of significance. The analysis was performed using StatsDirect statistical software version 2.3.4 for Windows.

Results

In neoplastic cells the pattern of TP staining was mostly cytoplasmic but occasionally also nuclear (Figure 1). Tumor fibroblastic stromal cells and inflammatory cells presented a cytoplasmic staining pattern (Figures 2 and 3). The immunoreactivity for tumor cells was mostly identified at the advancing front of the carcinomas (Figure 4), while for the TAS cells (stromal fibroblasts and inflammatory cells), staining was heterogeneous with no differences between the central and distal portion of the tumor. The proportion of the cells of the tumor tissue that expressed TP was 31.8% (21 cases), 60.6% (40 cases) and 48.5% (32 cases) for the tumor cells, stromal fibroblasts and tumor-associated inflammatory cells, respectively.

By considering the cases expressing TP in tumor cells as one group and all cases expressing TP in any subtype of TAS cells as another group, two distinct categories were obtained. The tumor-positive and the stroma-positive category. In the former category, 21 (31.82%) samples were positive, while 45 (68.18%) were evaluated as negative. In the latter category, 47 (71.21%) samples were positive and 19 (28.79%) were negative.

Statistical analysis revealed a significant association between TP expression in tumor cells and the age of the patients ($p=0.007$), as well as between stroma TP positive expression and poorly-differentiated towards moderately- and well-differentiated tumors ($p=0.033$) (Table I). Positive TP expression, either in the tumor or the stroma, was not statistically correlated to the gender of the patients or the tumor type, intestinal, diffuse or mixed.

No relationship was found between TP expression and any of the clinicopathological parameters under evaluation, when considering TP immunoreactivity for each group of TAS cells separately. The same results were obtained even when considering the tumors as TP-positive irrespective of the tissue localization of the enzyme (data not shown).

Positive expression of TP in tumor cells was also associated with poorer patient prognosis by Kaplan-Meier analysis (Figure 5). The mean survival time for patients with TP-positive tumors was 19 months, while for patients with TP-negative tumors was 31.5 months. These results were statistically significant according to log-rank and Wilcoxon (Pento-Prentice) tests ($p=0.015$) Positive expression of stroma TP was not associated with patient prognosis as analyzed by the Kaplan-Meier method.

No statistical analysis could be performed with respect to the stage of the patients, since most of the cases of our study were high stage tumors.

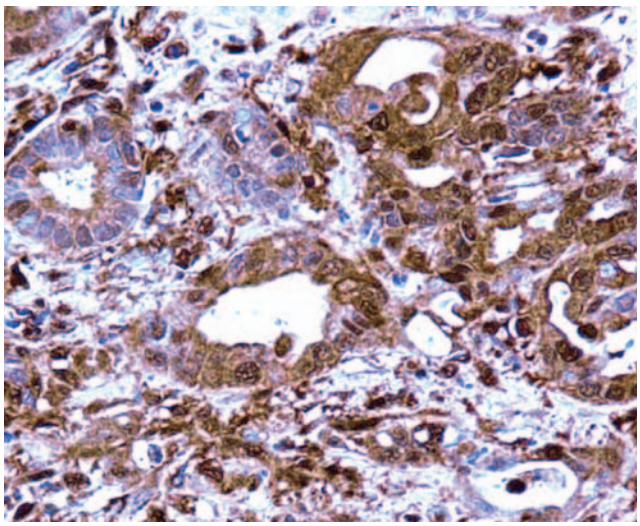

Figure 1. *Cytoplasmic and nuclear staining for thymidine phosphorylase (TP) in tumor cells and cytoplasmic staining in tumor-associated inflammatory cells (x 200).*

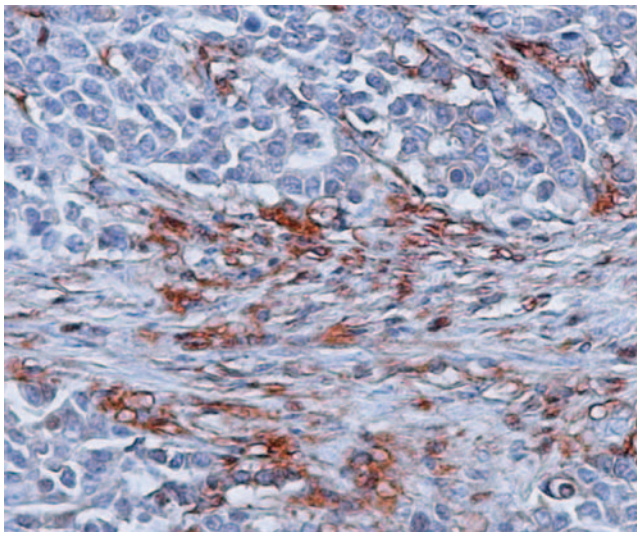

Figure 2. *Cytoplasmic staining in stromal fibroblasts (x 200).*

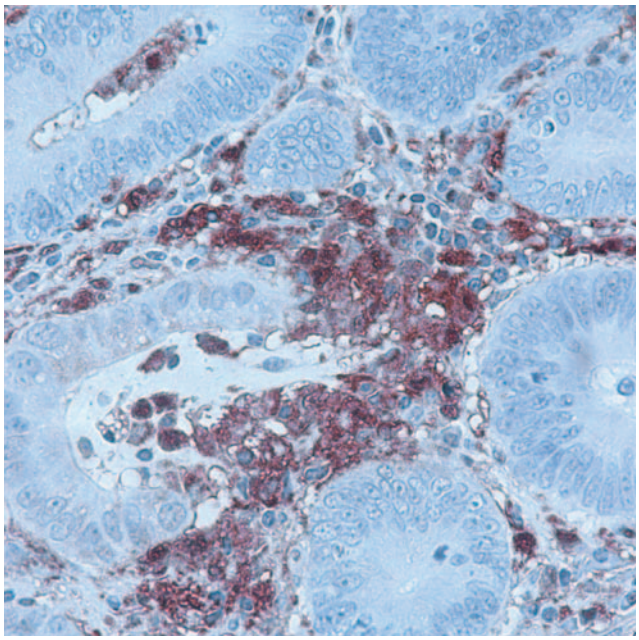

Figure 3. *Cytoplasmic staining in tumor-associated inflammatory cells (x400).*

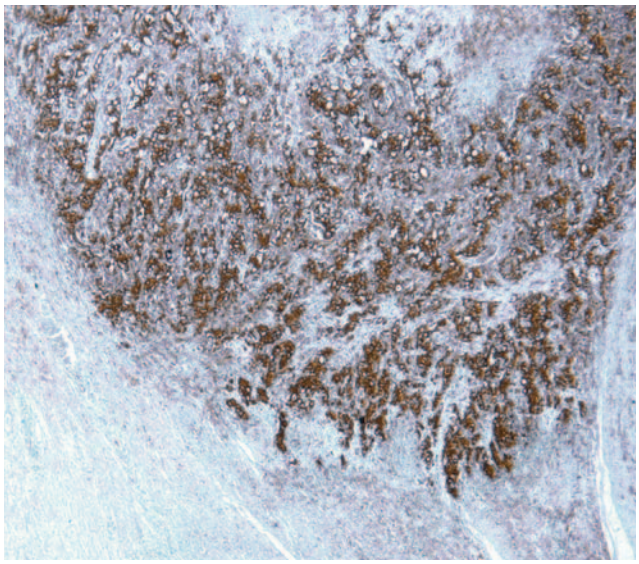

Figure 4. *Tumor-positive thymidine phosphorylase (TP) expression in the advancing front of the carcinoma (x100).*

Discussion

Tumor growth and metastasis is a biological process depending, not only on the properties of the neoplastic cells, but mostly on the interactions between cancer, stromal and adjacent infiltrating cells. Moreover, a tumor must continuously stimulate the growth of new capillary blood vessels for it to grow. These newly formed vessels facilitate the entry of tumor cells into the vasculature (18). It has been shown in several human solid tumors that this process of angiogenesis correlates with the probability of metastasis and tumor recurrence.

It has been suggested that the enzymatic products that derive from the action of TP, stimulate the chemotaxis of endothelial cells and possibly, other cells, causing angiogenesis. As such, TP is considered as an angiogenic factor (3).

The role of TP immunohistochemical expression in gastric carcinoma has been widely studied, but the results are

Table I. *Patient clinicopathological data and thymidine phosphorylase (TP) expression.*

	Tumor thymidine		*P*	Stromal thymidine		*P*
	21 (31.8%)	45 (68.2%)		47 (71.2%)	19 (28.8%)	
Characteristic	positive (+)	negative (–)		positive (+)	negative (–)	
Gender			NS			NS
M	13 (30.95%)	29 (69.05%)		30 (71.43%)	12 (28.57%)	
F	8 (33.33%)	16 (66.67%)		17 (70.83%)	7 (29.17%)	
Age			**0.007**			NS
36 – 70 yr	6 (16.21%)	31 (83.78%)		25 (66.67%)	12 (33.33%)	
71 – 88 yr	15 (50.00%)	14 (50.00%)		22 (75.86%)	7 (24.13%)	
Type			NS			NS
Intestinal	14 (41.17%)	20 (58.82%)		25 (73.53%)	9 (26.47%)	
Diffuse	4 (20%)	16 (80%)		13 (65%)	7 (35%)	
Mixed	3 (25.%)	9 (75%)		9 (75%)	3 (25%)	
Grade			NS			
High	2 (18.18%)	9 (81.82%)		9 (81.82%)	2 (18.18%)	
Medium	3 (21.43%)	11 (78.57%)		13 (92.86%)	1 (7.14%)	
Low	16 (39.02%)	25 (60.98%)	NS	25 (60.98%)	16 (39.02%)	**0.033**

controversial (19, 20). Some investigators found a significant association between the expression of TP and prognosis while others did not. It seems that this discrepancy was due to the fact that the proportion of well- to poorly-differentiated tumors varied between different studies. Moreover, no standard criteria are established concerning what should be considered positive TP reaction – the proportion of positively-stained tumor cells, tumor-associated stromal cells, or both? Should each different population of tumor associated stromal cells be considered separately or as a whole?

In our study, the proportion of tumor tissue cells that expressed TP was 32.8% (22 cases), 47.7% (32 cases), 74.6 % (50 cases) and 35.8% (24 cases) for the tumor cells, associated stromal cells and lymphocytes and macrophages, respectively. By considering TAS cells as a whole, the vast majority of the carcinomas (83.5%, 56 cases) were TP-positive. This is in accordance with the observations on breast and colorectal cancer (6, 7), indicating that TAS cells might be the main source of overall TP levels in the tumor tissue.

It is known that TP expression is induced by several cytokines and growth factors such as interleukin-1, tumor necrosis factor, basic fibroblast growth factor, interferon-α and interferon-γ, which are probably released from tumor-associated macrophages and reactive infiltrating inflammatory cells (21). Other studies showed that some of these cytokines also induced the expression of TP in tumor cells. Since the induction of TP expression in tumor cells and associated stromal cells might be the result of a common mechanism, it would probably be appropriate to consider all tumors expressing TP as TP-positive tumors, irrespective of the tissue localization of the enzyme. In the present study, no results were drawn concerning the relationship between TP expression and the clinicopathological parameters under evaluation, when considering the tumors as TP-positive irrespective of tissue localization. The same results were obtained by evaluating TP immunoreactivity independently for each category of TAS cells.

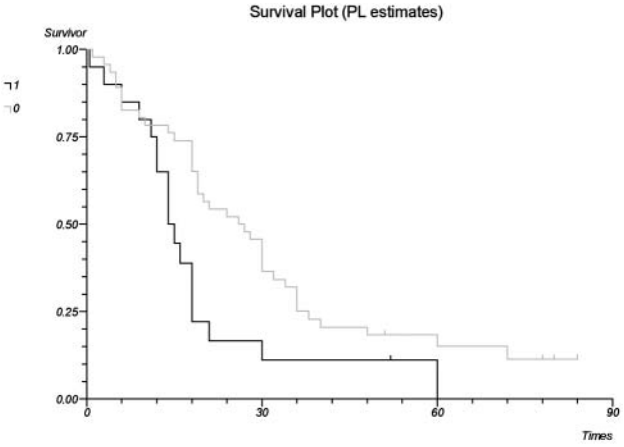

Figure 5. *Kaplan-Meier survival curve for tumor thymidine-positive (1) and tumor thymidine-negative (0) patients.*

However, the expression of TP in the group of stroma-positive tumors, was found to be related only to poorly-differentiated carcinomas. This result is in accordance with the observation of other studies, which postulated that TP expression in undifferentiated carcinomas is not correlated with any other clinicopathological parameter (22).

TP expression in tumor cells was found to be the most important prognostic indicator of tumor progression and

poor patient survival. This finding, in relation to the observation that, in our cases, TP immunoreactivity in gastric cancer cells was mostly identified in the advancing front of the tumor, strengthens the hypothesis that TP is involved in tumor progression. Such a correlation was not found in the stroma-positive tumors. One could speculate that this divergent significance of TP expression with respect to the tissue localization of the enzyme might be related to other functions of TP involved in tumor growth besides angiogenesis. It has been suggested that in addition to its angiogenic properties, TP confers resistance to apoptosis induced by hypoxia (5). Although the exact mechanisms are not yet elucidated, it seems that the degradation products of thymidine, thymine and 2-deoxy-D-ribose might be involved in the prevention of apoptosis.

In summary, we demonstrated that TP expression seems to be an important prognostic indicator in gastric cancer patients only when the enzyme is located in tumor cells as evaluated by immunohistochemistry. The different impact of the expression of TP in tumor cells and associated stromal cells, might be the result of the dual property of TP, as an angiogenic and anti-apoptotic factor.

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