

Significance of Thymidine Phosphorylase/Platelet-derived Endothelial Cell Growth Factor in Carcinoma of the Papilla of Vater

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The expression of thymidine phosphorylase (TP) in carcinoma of the papilla of Vater was studied to clarify its significance in tumor progression and in determining prognosis. Fifty-nine cases of surgically resected carcinoma of the papilla of Vater were studied. Immunohistochemical staining was performed to evaluate the expression of TP, microvessel count and p53 overexpression. TP expression was demonstrated in tumor cells in 62.7% (37/59) of the cases. A higher frequency of regional lymph node metastasis was found in TP-positive tumors than in TP-negative tumors ($P=0.006$). TP-positive tumors were more advanced than TP-negative tumors with regard to clinical stage ($P=0.035$). TP-positive tumors had significantly higher microvessel density (27.6 ± 10.1) than TP-negative tumors (20.4 ± 10.0 , $P=0.01$). Moreover, TP expression was significantly correlated with a poor prognosis ($P=0.02$). These suggest that in carcinoma of the papilla of Vater, TP production by tumor cells is correlated with tumor progression through its regulatory effect on neovascularization.

Key words: TP — Microvessel density — p53 — Prognosis

Carcinoma of the papilla of Vater is a malignancy that occurs much less often than pancreatic carcinoma. However, it accounts for nearly 40% of all resected carcinomas in the periampullary region, and is second only to pancreatic carcinoma.^{1–4} It has a better prognosis than pancreatic carcinoma. However, the molecular characteristics of this malignancy have not been fully documented.^{5–7} To our knowledge, there has been no study on the expression of thymidine phosphorylase (TP) in carcinoma of the papilla of Vater, and the role of TP has not yet been elucidated.

New vascular formation is necessary for the growth and proliferation of solid tumors.^{8,9} Folkman¹⁰ claimed that neovascularization was necessary for the invasion and metastasis of malignant tumors. The process of neovascularization is regulated by various angiogenic factors, including promoting factors and inhibiting factors. Platelet-derived endothelial cell growth factor (PD-ECGF) is an angiogenic factor that stimulates endothelial cell growth and chemotaxis *in vitro* and angiogenesis *in vivo*.^{11–13} TP is an enzyme that is involved in the process of nucleotide metabolism.¹⁴ It catalyzes the metabolism of thymidine. Recently, it has been demonstrated that TP is identical in structure to PD-ECGF.^{14,15} The dephosphorylated end product of thymidine metabolism, 2-deoxyribose, is responsible for its angiogenic activity.

It has been reported that the serum concentration of TP in patients with carcinoma is higher than that in normal controls.^{16,17} Moreover, recent studies have shown that immunohistochemical expression of TP in tumor tissues is correlated with microvessel formation, and has prognostic significance in human malignancies, e.g., in the colon,^{18,19} stomach,^{20–22} breast,²³ bladder,^{24,25} ovary,²⁶ lung,²⁷ and pancreas.²⁸

In this study, we investigated the expression of TP by an immunohistochemical method in carcinoma of the papilla of Vater to clarify its possible role in tumor progression. The expression was compared with microvessel density (MVD), p53 protein overexpression and other clinicopathologic factors, as well as the patients' survival.

MATERIALS AND METHODS

Patients and samples Fifty-nine cases of carcinoma of the papilla of Vater were studied. Patients were treated at the Department of Surgery, Tokyo University, or the Department of Surgery, Tokyo Metropolitan Institute of Gerontology, from 1970 to 1997. Pancreatoduodenectomy (including pylorus-preserving pancreatoduodenectomy in two cases) was performed in all patients. Formalin-fixed, paraffin-embedded samples were available for all cases. The mean age of the patients was 64 years (range 42 to 85) (Table I). There were 37 men and 22 women. TNM stage was classified according to the American Joint Committee on Cancer Staging Manual.²⁹ Eight cases were in

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Table I. Clinical Characteristics of Patients

Age (Years)	Mean	64
	Range	42–85
Gender	Male	37
	Female	22
TNM stage (AJCC)	I	8
	II	14
	III	31
	IV	6
Operation	PD ^{a)}	57
	PpPD ^{b)}	2

a) Pancreatoduodenectomy.

b) Pylorus preserving pancreatoduodenectomy.

stage I, 14 were in stage II, 31 were in stage III, and 6 were in stage IV. Five-micrometer-thick serial sections were cut from paraffin-embedded blocks for hematoxylin and eosin (HE) staining and immunohistochemical staining.

Immunohistochemical staining for TP For immunostaining of TP, the streptavidin-biotin complex method was used, following the manufacturer's instructions. Briefly, after deparaffinization, antigen retrieval in citrate buffer (pH 6) was performed by heating in a microwave oven at 100°C for 10 min. Endogenous biotin activity was blocked using a biotin blocking kit (Vector Laboratories, Inc., Burlingame, CA). After washing with phosphate-buffered saline (PBS), incubation with 3% skim milk was performed to block any nonspecific antibody binding. Endogenous peroxidase activity was blocked in methanol with 0.3% H₂O₂ for 30 min. Samples were incubated at 4°C overnight with mouse anti-human TP monoclonal antibody 654-1 (Nippon Roche Research Center, Kamakura) at a dilution of 1:200. The monoclonal antibody is specific for TP protein.³⁰⁾ Samples were then incubated with biotinylated anti-mouse antibody for 30 min at room temperature. After incubation with avidin-biotin complex (Vector Laboratories, Inc.) for another 30 min, samples were developed with 3,3'-diaminobenzidine (DAB, Vector Laboratories, Inc.). Finally, samples were counterstained with hematoxylin and mounted. A negative control was created by substituting the primary antibody with normal mouse IgG.

Immunohistochemical staining and evaluation for microvessel formation Microvessel staining was performed using mouse anti-factor VIII-related antigen (F-VIII RAg) monoclonal antibody F8/86 (Dako A/S, Glostrup, Denmark) in a dilution of 1:50. The procedure was the same as that for immunostaining for TP, except for omission of incubation with skim milk. Microvessels were counted according to the method reported by Maeda *et al.*,²⁰⁾ with minor modifications. Briefly, the section was

first investigated under low magnification (×5 objective lens) to identify the area with the highest density of microvessels. The area with the highest density of microvessels was then highlighted under high magnification (×20 objective lens, about 1 mm²/field), and the number of microvessels was considered the MVD of the tumor. Any brown-stained structure that stood by itself was considered a single vessel.

Immunostaining for p53 protein Mouse anti-human monoclonal antibody Do7 (Dako A/S; diluted to 1:100) was used to stain p53 protein. The procedure was the same as that for TP staining, except for omission of incubation with skim milk. A cell line with known p53 overexpression was used as a positive control. Staining was evaluated using an Image Cytometer CAS 200R (Cell Analysis System Inc., Elmhurst, IL). Briefly, 20 fields were randomly selected in each stained section, and the percentage of stained cells in each field was counted. An average percentage of the 20 fields of above 10% was considered positive.

Statistical analysis The χ^2 test was used to analyze the relationship between TP expression and other clinicopathologic parameters and p53 overexpression. Student's *t* test was used to analyze the relationship between MVD and TP expression, as well as other variables. Survival curves were plotted using the Kaplan-Meier method, and the log-rank test was used to evaluate the significance of differences. Cox's proportional hazard model was used to assess the effects of various factors on survival. A *P* value of less than 0.05 was considered significant.

RESULTS

Immunostaining for TP Normal duodenal epithelium was scarcely stained by antibody 654-1. In only a few cases was normal epithelium stained (less than 10% of all tumor cells). In contrast, TP was strongly expressed in tumor cells. Staining was mostly observed in the cytoplasm of tumor cells (Fig. 1), and in some cases, also in the nuclei. We considered the tumor as negative for TP if less than 10% of tumor cells was stained. Those in which more than 10% was stained were considered to be positive. In all, 62.7% (37/59) of cases were positive for TP. As regards the stromal cells, TP expression was found in some stromal cells within normal epithelia. Tumor-infiltrating stromal cells were positive in 83.1% of cases (49/59) (Fig. 1). Moreover, in all cases with staining of TP in tumor cells, stromal cells were also positive. However, in some cases stromal cells were stained for TP even though no tumor cells were stained. The expressions of TP in tumor cells and stromal cells were significantly correlated (*P*<0.001).

Correlations between TP expression and clinicopathologic variables The correlations between TP expression and clinicopathologic factors are shown in Table II. There

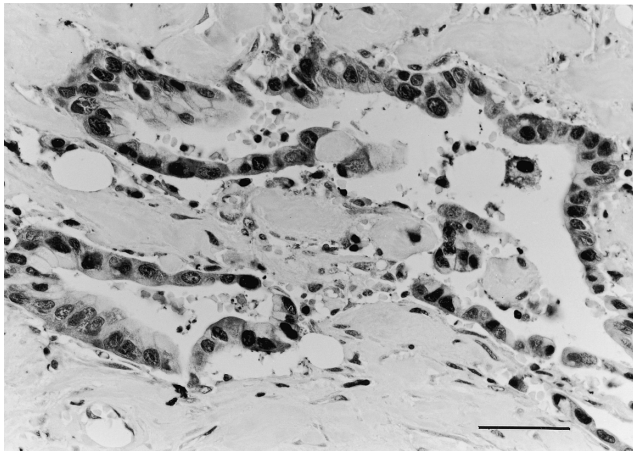


Fig. 1. Immunohistochemical staining of TP in carcinoma tissues of the papilla of Vater. Strong cytoplasmic staining of tumor cells was detected. Staining was also detected in some infiltrating cells. (size bar=50 μ m)

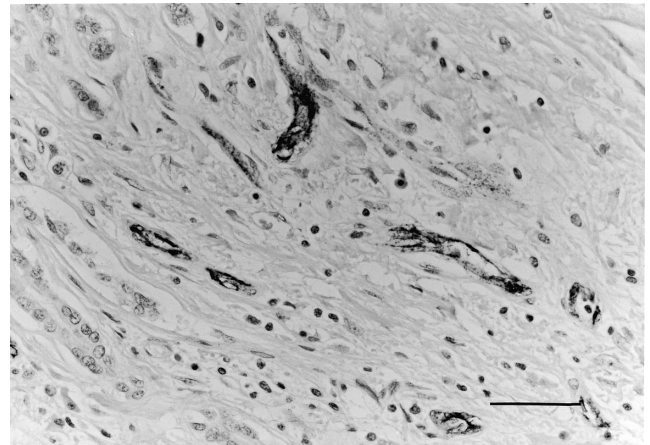


Fig. 2. Immunohistochemical staining of microvessels by anti-factor VIII-related antigen. The highest MVD was usually detected in tissues surrounding tumor nests. (size bar=50 μ m)

Table II. Correlations between TP and Clinicopathologic Variables

Variables		TP negative	TP positive	<i>P</i>
Venous invasion	(-)	14	17	0.293
	(+)	8	20	
Lymphatic invasion	(-)	10	11	0.665
	(+)	12	26	
Neural invasion	(-)	13	18	0.138
	(+)	9	19	
Ulceration	(-)	10	24	0.145
	(+)	12	13	
p53 overexpression	(-)	13	20	0.706
	(+)	9	17	
Pancreatic invasion	(-)	13	20	0.789
	(+)	9	17	
Lymph node metastasis	(-)	14	10	0.006
	(+)	8	27	
TNM stage (AJCC)	I	5	3	0.035
	II	7	7	
	III	8	23	
	IV	2	4	

was no significant association between TP expression and tumor size, lymphatic invasion, venous invasion or neural invasion. However, a significantly higher frequency of regional lymph node metastasis was found in TP-positive tumors than in TP-negative tumors ($P=0.006$). TP-positive tumors were also more advanced with regard to stage than TP-negative tumors. When tumors were divided into a slightly progressed group (including tumors in stages I and

Table III. Relation between TP Expression and MVD

TP expression	No.	Mean \pm SD ^{a)}	<i>P</i>
Negative	22	20.4 \pm 10.0	0.01
Positive	37	27.6 \pm 10.1	

a) SD, standard deviation.

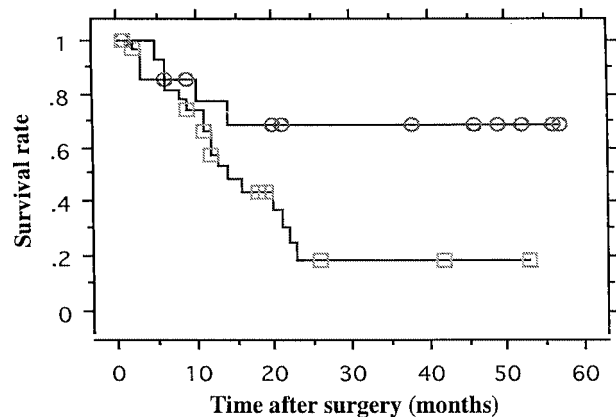


Fig. 3. Overall Kaplan-Meier survival curve with respect to TP expression in tumor cells of patients with carcinoma of the papilla of Vater ($P=0.02$). \circ TP-negative cases, \square TP-positive cases.

II) and a highly progressed group (including tumors in stages III and IV), the highly progressed group contained significantly more TP-positive tumors ($P=0.035$). p53 protein was exclusively stained in the nuclei of tumor cells.

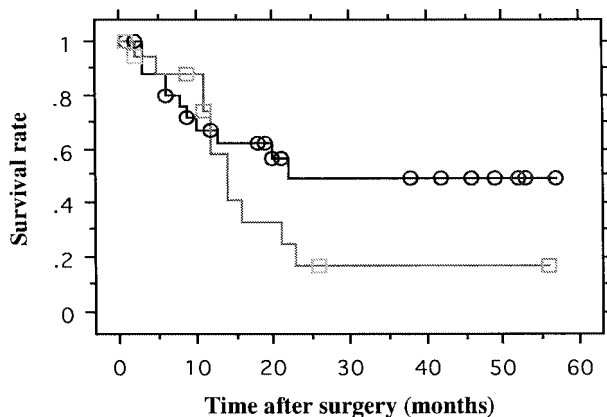


Fig. 4. Overall Kaplan-Meier survival curve with respect to MVD of patients with carcinoma of the papilla of Vater ($P=0.18$). ○ low-MVD cases, □ high-MVD cases.

TP expression did not correlate with p53 overexpression. No correlation was found between the staining in tumor infiltrating cells and any of the clinicopathologic variables (data not shown).

Relationships between MVD and TP expression and clinicopathologic variables Microvessel formation was mostly found in the tissues surrounding tumor tissues rather than in the center of tumors (Fig. 2). The mean MVD in TP-positive tumors (27.6 ± 10.1) was significantly higher than that in TP-negative tumors (20.4 ± 10.0 , $P=0.01$, Table III). p53-positive tumors had higher MVD, but this difference was not significant.

Prognostic value of TP expression and MVD There was a significant correlation between TP expression and overall postoperative survival (Fig. 3). Patients with TP-positive tumors had a shorter postoperative survival than those with TP-negative tumors ($P=0.02$). When tumors were stratified by stage into slightly progressed and highly progressed groups, patients with TP-positive tumors had a worse prognosis than those with TP-negative tumors in each group, but these differences were not significant by the log-rank test. In evaluating the correlation between MVD and prognosis, tumors were divided into a low-MVD group and a high-MVD group based on the median value of MVD (25.5) for all of the cases. Patients in the high-MVD group had a shorter postoperative survival than those in the low-MVD group, but this difference was not significant ($P=0.18$, Fig. 4). Univariate analysis showed that TP, lymph node metastasis and tumor stage were significantly related to a poor prognosis. However, a multivariate analysis using Cox's proportional hazard model showed that, except for tumor size, pancreatic invasion, and lymph node metastasis, neither TP nor MVD was an independent prognostic factor.

DISCUSSION

Recently, great attention has been paid to TP as a key factor in promoting tumor angiogenesis in several kinds of malignancies. Serum levels of TP have been shown to be increased in patients with cancers compared to those in healthy controls.^{31, 32} Our current study is the first concerning the expression of TP in carcinoma of the papilla of Vater in a relatively large series. Epithelial cells within normal duodenal mucosa were scarcely stained for TP. In contrast, TP was strongly stained in tumor cells, being mostly confined to the cytoplasm.

In the present study, regional lymph node metastasis was significantly more frequent in tumors that expressed TP. Tumors that were positive for TP were in more advanced stages and TP expression was significantly correlated with a worse prognosis. These findings suggest that TP plays a positive role in tumor progression in carcinoma of the papilla of Vater. Our results are consistent with previous reports dealing with other malignancies.^{21, 32-37} Moreover, TP-positive tumors had a significantly higher MVD than TP-negative tumors, which supports the idea that TP plays a role in promoting tumor progression through its regulation of tumor angiogenesis, as suggested by other authors.^{20, 21}

The roles of various types of cells in the regulation of the expression of TP within carcinoma is still unclear, as pointed out by Folkman.³⁸ The effect of TP on microvessel formation seems to vary in tumors of different origin, and its location within tumors, as demonstrated by immunohistochemical methods remains controversial. Some authors observed the expression of TP mainly in tumor-infiltrating stromal cells, and only in a minority of cancer cells.^{19, 39, 40} On the other hand, other authors reported that TP is strongly expressed in cancer cells, and also expressed in infiltrating cells within carcinomas to some degree.^{18, 21, 35-37} In our study, TP was expressed in both tumor and infiltrating cells (fibroblasts or macrophages). Their stainings were correlated. However, tumor-infiltrating cells were stained in some cases even when no expression was detected in tumor cells. Since TP is also expressed in some infiltrating cells within normal epithelia to a mild degree, TP expressed in tumor-infiltrating cells may be related more to inflammation than to tumor vascular formation. Moreover, we found that the expression of TP in tumor cells, but not that in tumor-infiltrating stromal cells, was correlated with tumor progression. This suggests that tumor cells play a key role in producing TP in carcinoma of the papilla of Vater.

In our study, although MVD was correlated with the expression of TP, the TP-negative group included some tumors with high MVD. Since it is unlikely that a single factor is responsible for the process of angiogenesis, as has been pointed out by other authors,^{41, 42} there may be

other angiogenic factors in addition to TP that can regulate microvessel formation in carcinoma of the papilla of Vater, as in other malignancies.^{19, 43)}

In the present study, TP was correlated with lymph node metastasis. TP may play a role in lymph node metastasis because new vascular formation provides necessary nutrients for tumor growth, and when tumors become large enough they first invade lymph vessels due to the relative weakness of lymph vessels. It is also possible that TP promotes the formation of lymph vessels. Increased lymph vessels may make it easier for tumors to invade the lymphatic circulation. In multivariate analysis, expression of TP was not independent of regional lymph node metastasis in determining prognosis. We speculate that TP is more closely correlated with lymph metastasis in this malignancy.

TP was significantly correlated with the prognosis, though MVD was not. This suggests that TP may affect tumor progression through unknown mechanisms other than angiogenesis.

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