Jpn. J. Cancer Res. 87, 288–295, March 1996

Expression of Thymidine Phosphorylase in Human Gastric Carcinoma

Yuji Takebayashi,[1,2] Kazutaka Miyadera,[1,4] Shin-ichi Akiyama,[1,5] Shuichi Hokita,[2] Kazutaka Yamada,[2] Suminori Akiba,[3] Yuji Yamada,[4] Tomoyuki Sumizawa[1] and Takashi Aikou[2]

[1]*Department of Cancer Chemotherapy, Institute for Cancer Research,* [2]*First Department of Surgery,* [3]*Department of Public Health, Faculty of Medicine, Kagoshima University, Sakuragaoka 8-35-1, Kagoshima 890 and* [4]*Taiho Pharmaceutical Co. Ltd., Misugidai 1-27, Hanno, Saitama 357*

The activity of thymidine phosphorylase (dThdPase) has been reported to increase in several types of malignant tumors. Experimental evidence has shown that dThdPase is identical to platelet-derived endothelial cell growth factor, and that dThdPase has angiogenic activity. We examined the expression of dThdPase to investigate whether the expression of dThdPase correlates with angiogenesis, clinicopathologic features and the prognosis of patients with human gastric carcinomas. Microvessels were assessed by immunostaining endothelial cells for factor VIII. We counted microvessels in the tumors of 158 patients whose tumors were completely removed surgically. Microvessels were counted in a ×400 field in the most active areas of neovascularization. We purified a monoclonal antibody (TMA-1) against dThdPase and studied the expression of dThdPase using TMA-1 in the same serial sections as those used for the detection of factor VIII. The correlation between angiogenesis and dThdPase, and the clinicopathological significance of dThdPase, in patients with gastric carcinoma were examined. The positive expression of dThdPase was more frequent ($P<0.001$) in gastric carcinomas (67/158, 43.4%) than that in normal tissues (12/158, 7.6%). The average microvessel count in dThdPase-positive gastric carcinomas was higher ($P<0.001$) than that in dThdPase-negative carcinomas. The percentage of gastric carcinoma cells expressing dThdPase was significantly correlated with the microvessel count ($P<0.001$). Further, the average size of dThdPase-positive carcinomas was significantly larger ($P<0.001$) than that of negative carcinomas and the mean microvessel count in dThdPase-positive gastric carcinomas was also significantly higher ($P<0.001$) than that in dThdPase-negative carcinomas. There was a significant correlation between the positive expression of dThdPase and microvessel count ($P<0.001$) or lymph node metastasis ($P=0.013$) by multivariate logistic analysis. Further, patients with dThdPase-positive carcinoma showed a significantly worse prognosis than those with dThdPase-negative carcinoma overall and in stage III. These findings indicate that the expression of dThdPase in gastric carcinomas is related to progression and metastasis, and this enzyme affects the prognosis of some patients with the disease.

Key words: Thymidine phosphorylase — Immunohistochemistry — Gastric carcinoma — Angiogenic factor — Platelet-derived endothelial cell growth factor

Thymidine phosphorylase (dThdPase; EC 2.4.2.4) catalyzes the reversible phosphorolysis of thymidine, deoxyuridine and their analogs to their respective bases and 2-deoxyribose-1-phosphate.[1-3] dThdPase also catalyzes the transfer of deoxyribose from one deoxynucleoside to another base to form a second deoxynucleoside.[4-6] In mammals, dThdPase consists of two identical subunits with a molecular weight of 55,000 daltons.[7] We have previously shown that dThdPase is identical to platelet-derived endothelial cell growth factor (PD-ECGF).[8-10] PD-ECGF stimulates chemotaxis and [^{3}H]thymidine incorporation by endothelial cells *in vitro* and has angiogenic activity *in vivo*.[11,12] Recently, we have demonstrated that the enzymatic activity of dThdPase is indispensable for the angiogenic activity of dThdPase.[13] dThdPase activity has been reported to increase in a variety of malignant tumors compared with the adjacent normal tissues.[4,14-17] Expression of dThdPase has been reported to be significantly higher in colorectal carcinoma than in normal colorectal tissue and colorectal adenoma, and its activity has been correlated with the expression of thrombomodulin, an endothelial cell marker.[17]

Experimental evidence has shown that tumor growth is dependent on angiogenesis.[18,19] When tumors reach a size of a few millimeters, new capillaries penetrate them, allowing rapid growth. These new vessels facilitate the entry of tumor cells into the vasculature and their subsequent metastasis, so that angiogenesis correlates with the probability of metastases.[20-23]

In cutaneous melanoma, the intensity of angiogenesis is correlated with the probability of metastasis.[22] In breast carcinoma, a significant correlation between the proportion of metastasis and the microvessel density has been demonstrated,[24] and microvessel density was

[5] To whom all correspondence should be addressed.

an independent prognostic factor.[25, 26] Similar findings have been reported concerning prostate[27] and lung carcinoma.[28, 29]

In the present study we examined the expression of the angiogenic factor dThdPase in primary gastric carcinomas to determine whether dThdPase expression is correlated with the clinicopathologic variables of the disease.

MATERIALS AND METHODS

Patients The characteristic clinical features of the 158 patients with gastric carcinomas investigated in this study are summarized in Table I. All patients (112 males, 46 females; average age, 63 years, ranging from 46 to 82 years) were selected consecutively from January 1985 to December 1991, and had received no prior chemotherapy or irradiation. Tumors of 166 patients were completely removed surgically at the First Department of Surgery, Kagoshima University Hospital. All routine sections were carefully investigated to identify venous or lymphatic invasion. The specimens of carcinomas and normal tissues obtained from these patients were immunohistochemically stained using monoclonal antibody against dThdPase. Clinicopathological findings were described according to the general rules for gastric cancer study in surgery and pathology.[30]

Preparation of monoclonal antibody against dThdPase (TMA-1) GST fusion product containing 244 amino acid residues (amino acids 7 to 250) from the NH_2-terminus of PD-ECGF was produced in bacteria and partially purified. A DNA fragment containing about the 5′ half of the coding sequence of the PD-ECGF was generated from the *Xma* I digestion of plasmid pPL816 carrying the full length PD-ECGF cDNA, kindly supplied by Drs. K. Miyazono and C. H. Heldin (Ludwig Institute for Cancer Research, Uppsala, Sweden). The resulting 0.8 kb fragment was purified and inserted into plasmid pGEX-2T (Pharmacia, Uppsala, Sweden). Cultures of *Escherichia coli* cells were incubated with isopropyl-β-D thiogalactopyranoside for 4 h at 37°C, pelleted, and lysed with 2× sample buffer (125 m*M* Tris-HCl, pH 6.8, 4% sodium dodecyl sulfate, 20% glycerol, 10% 2-mercaptoethanol, and 0.002% bromophenol blue). The lysate was subjected to preparative electrophoresis (Nihon Eido, Tokyo, NA-1800 type) and separated. Fractions of 0.3 ml were collected and checked by electrophoresis (PAGE) with Coomassie blue staining. The five fractions that mainly contained 53 kDa-GST fusion product were collected (total protein amount was about 6.4 mg) and used for the preparation of monoclonal antibody against dThdPase. The 55 kDa protein was detected in the lysates of cells transfected with dThdPase,[13] human gastric cancers and adjacent non-neoplastic tissues by immunoblotting with this antibody (data not shown).

Table I. Relationship between dThdPase Expression and Clinicopathologic Variables in Gastric Carcinoma

Variable	dThdPase evaluation (%)		
	negative (n=91)	positive (n=67)	*P* values
Sex			0.721
Female	28 (60.9)	18 (39.1)	
Male	63 (56.3)	49 (43.7)	
Tumor location			0.394
C	31 (52.2)	28 (47.8)	
M	32 (65.3)	17 (34.7)	
A	28 (56.0)	22 (44.0)	
Histologic type			0.363
well	41 (66.1)	21 (34.9)	
mode	28 (52.8)	25 (47.2)	
poor	17 (50.0)	17 (50.0)	
mucinous	2 (66.7)	1 (33.3)	
signet	3 (50.0)	3 (50.0)	
Wall invasion			$P<0.001$
m/sm	58 (79.5)	15 (20.5)	
mp	8 (42.1)	11 (57.9)	
ss/se	22 (37.9)	36 (62.1)	
sei	3 (37.5)	5 (62.5)	
Lymph node metastasis			$P<0.001$
negative	77 (72.0)	30 (28.0)	
positive	14 (27.5)	37 (72.5)	
Lymphatic invasion			$P<0.001$
negative	59 (71.1)	24 (28.9)	
positive	32 (42.7)	43 (57.3)	
Venous invasion			0.084
negative	81 (60.4)	53 (39.6)	
positive	32 (42.7)	43 (57.3)	
Stage			$P<0.001$
I	71 (77.2)	21 (22.8)	
II	9 (28.1)	23 (71.9)	
III	8 (34.8)	15 (65.2)	
IV	3 (27.3)	8 (72.7)	

Tissue staining and evaluation of stained sections Samples were fixed with 10% formaldehyde in phosphate-buffered saline (PBS), embedded in paraffin, and cut into 3 μm thick sections. The sections were deparaffinized with xylene and dehydrated with 98% ethanol. Endogenous peroxidase was blocked by immersing the slides in 0.3% hydrogen peroxidase in absolute methanol for 20 min at room temperature. After having been washed three times with PBS for 5 min, the sections were blocked by soaking in PBS containing 1% bovine serum albumin for 20 min at room temperature. The blocked sections were incubated overnight at 4°C with TMA-1 diluted 500-fold with PBS. The following morning, the slides were incubated for 30 min with biotinylated anti-mouse IgG diluted 100-fold with PBS at room temperature. The sections were washed 3 times in PBS for 5 min, then

incubated with streptavidin-horseradish peroxidase complex diluted 100-fold with PBS for 30 min.[31] They were again washed three times in PBS for 5 min, then incubated with 0.5 mg/ml diaminobenzidine and 0.03% (v/v) H_2O_2 in PBS for 7 min and finally counterstained with hematoxylin prior to mounting. Serial sections were also incubated with rabbit antiserum against human von Willebrand factor (Dako Polyclonal, Dako Corporation, Santa Barbara, CA) diluted 1:200 with PBS containing 5% goat serum. Antibody binding was detected by sequential incubation with biotinylated goat anti-rabbit serum and streptavidin-peroxidase complex. Otherwise, the experimental protocol was as described for dThdPase.

For microscopic analysis of sections stained with TMA-1, we examined 200 cells to determine positivity for dThdPase. We decided that the specimens should be regarded as dThdPase-negative when less than 5% of the cells were stained, and positive when more than 5% of the cells were stained, since dThdPase-expressing cells amounted to less than 5% in most of the normal tissues examined (Fig. 1).

Microvessel counts were obtained by first screening for areas of intense neovascularization at low power (40× and 100×). Areas with the highest number of factor VIII-positive microvessels were then examined at higher power (400×) to obtain accurate microvessel counts (Fig. 2A). The latter, as well as the evaluation of dThdPase, were assessed without knowledge of the patient's clinicopathological factors, and were performed by two investigators simultaneously (Y. T. and K. M.).

Statistical analysis Two-sided statistical differences between proportions were examined using logistic analysis. Student's t test was conducted to evaluate significance of differences in the means of age, tumor size and microvessel count. Survival analysis was performed using the Kaplan-Meier method[32] and the significance of the differences between the curves was tested using the generalized Wilcoxon test.[33] The P-values were calculated and values less than 0.05 were considered significant.

RESULTS

Expression of dThdPase in normal mucosa and gastric carcinoma Most of the normal gastric mucosal cells were not stained with TMA-1. In contrast, the cytoplasm of

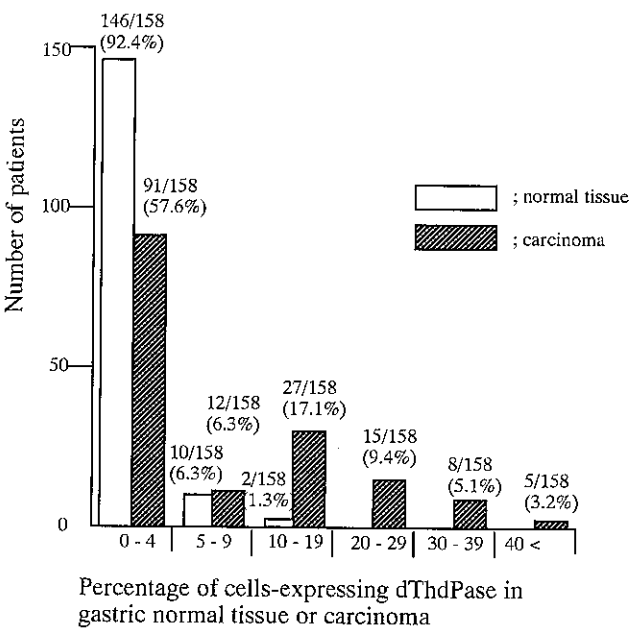

Fig. 1. Distribution of number of patients according to the percentage of cells expressing dThdPase.

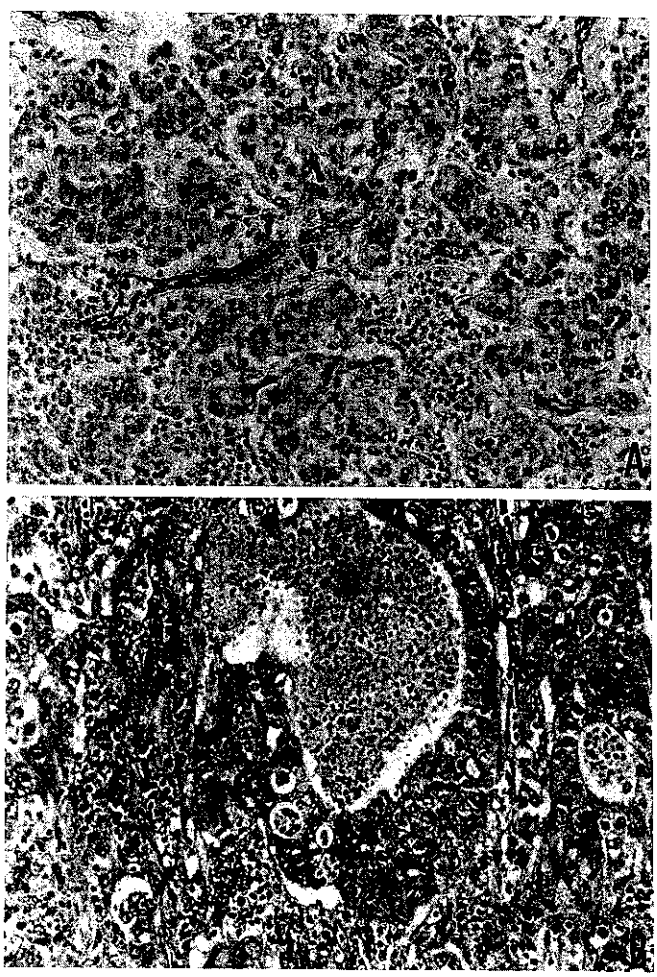

Fig. 2. (A) Microvessels detected by a factor VIII polyclonal antibody on a ×400 field. (B) dThdPase immunoreaction: high-level expression of dThdPase was detected in gastric carcinoma cells.

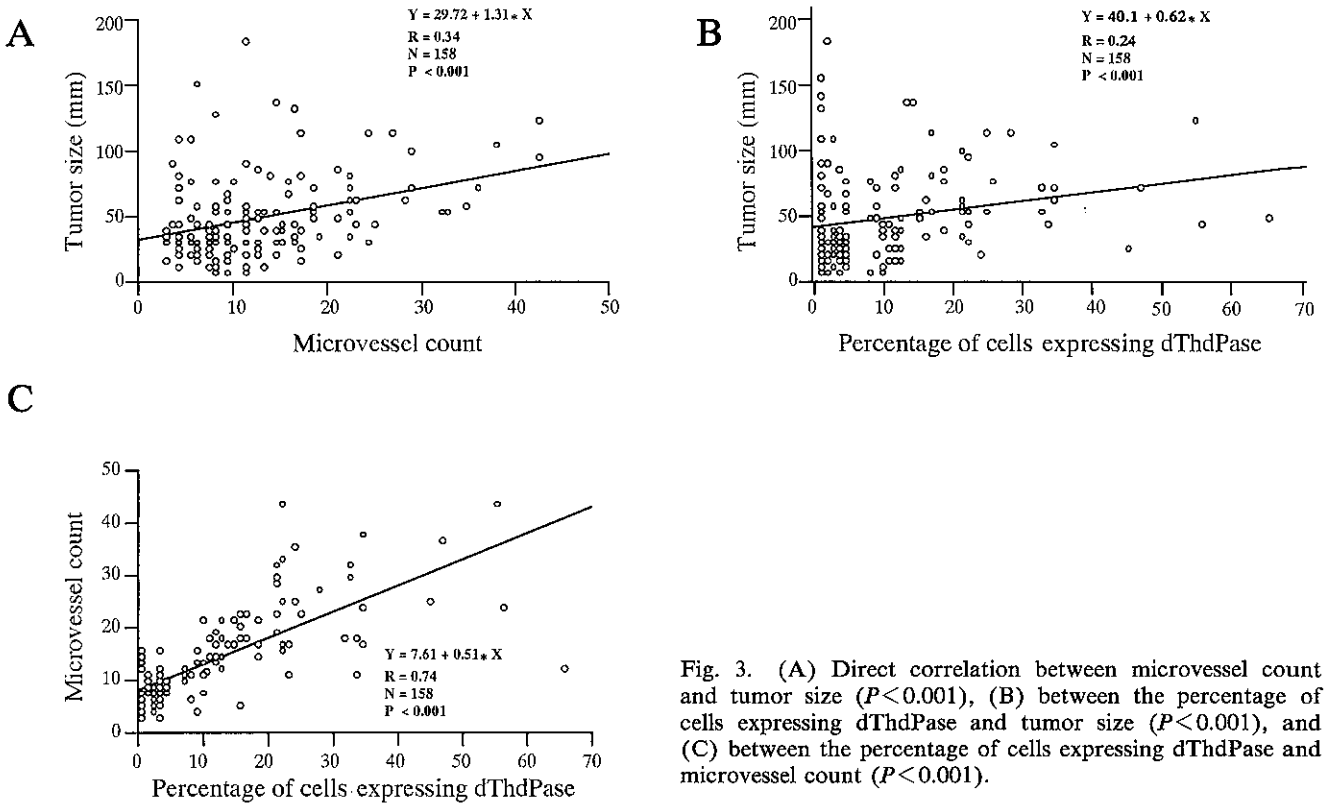

Fig. 3. (A) Direct correlation between microvessel count and tumor size ($P<0.001$), (B) between the percentage of cells expressing dThdPase and tumor size ($P<0.001$), and (C) between the percentage of cells expressing dThdPase and microvessel count ($P<0.001$).

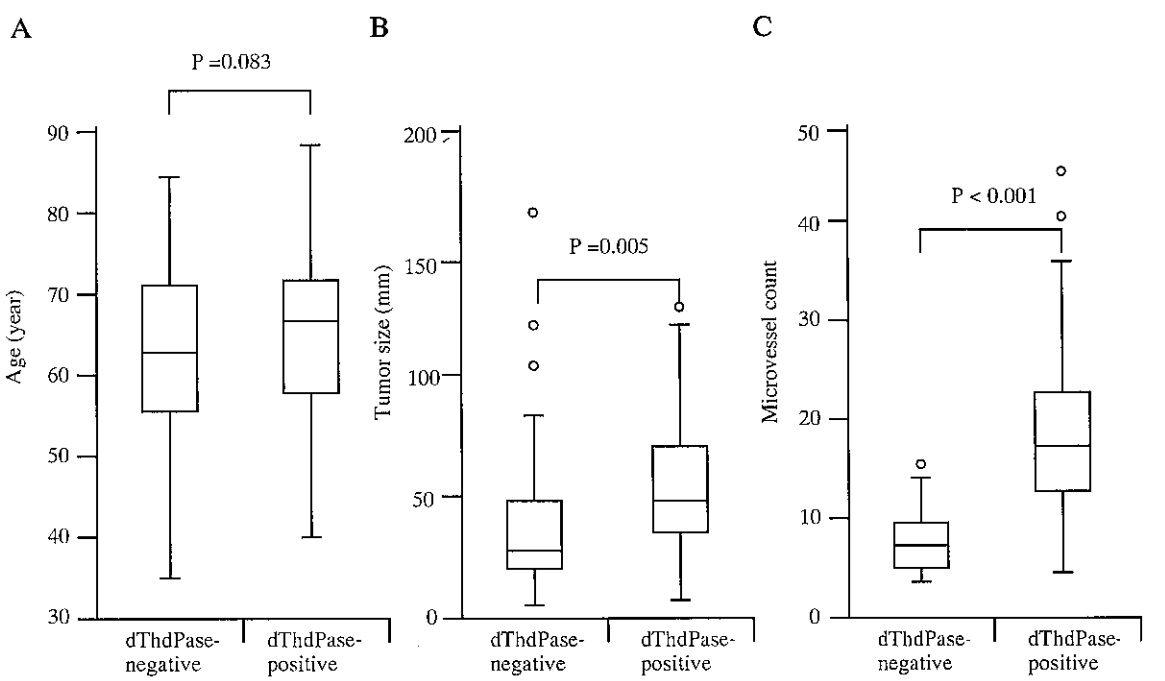

Fig. 4. Comparison of age (A), tumor size (B) or microvessel count (C) assessed by immunohistochemistry using TMA-1. The boxes correspond to the interquartile ranges, with the lower boundary of the box representing the 25th percentile and the upper boundary, the 75th percentile. The lines in the boxes represent the mean values. The whiskers represent the 5th and 95th percentiles.

Table II. Relationship between dThdPase Expression and Clinicopathologic Variables by Multivariate Logistic Analysis

Variable	Multivariate analysis		
	Odds ratio	95% confidence interval	*P* value
Size (mm)	1.01	0.99–1.03	0.203
Wall invasion (m/sm vs. mp, ss/se, sei)	1.44	0.88–2.35	0.379
Lymph node metastasis (absent vs. present)	3.35	1.28–8.76	0.013
Lymphatic invasion (absent vs. present)	0.76	0.27–2.10	0.590
Microvessel count (number)	1.24	1.12–1.37	<0.001

the cells of many gastric carcinoma cells was strongly stained (Fig. 2B). Since the percentage of cells expressing dThdPase was less than 5% of 200 cells in 92.4% of normal tissues, specimens were regarded as positive when more than 5% of the examined cells were stained (Fig. 1). We found that 42% of gastric carcinomas had higher percentages than adjacent normal mucosa (Fig. 1).

Relationship between tumor size, microvessel count and the expression of dThdPase in gastric carcinomas We plotted the tumor size against microvessel count (Fig. 3A) or the percentage of cells expressing dThdPase (Fig. 3B). There were significant correlations between the tumor size and microvessel count or percentage of cells expressing dThdPase ($P<0.001$). We also plotted the percentage of cells expressing dThdPase against the microvessel count (Fig. 3C). A significant correlation between the percentage of cells expressing dThdPase and the microvessel count was observed ($P<0.001$). Further, the average size of dThdPase-positive carcinomas was significantly larger ($P<0.001$) than that of negative carcinomas, and the mean microvessel count in dThdPase-positive gastric carcinomas was also significantly higher ($P<0.001$) than that in negative carcinomas (Fig. 4B, C).

Relationship between clinicopathologic factors and dThdPase expression Although no significant correlation was found between dThdPase expression and age, sex, tumor location, histologic type or venous invasion, significant correlations with other pathologic and clinical findings were observed by univariate analysis (Table I, Fig. 4A, B). Compared to dThdPase-negative carcinomas, tumors which stained positive invaded more deeply than negative tumors ($P<0.001$), and had a higher frequency of lymph node metastasis ($P<0.001$). Expression of dThdPase was significantly higher in carcinomas with lymphatic invasion ($P<0.001$). As regards stages, a significant difference of dThdPase positivity was observed only between stage I and II ($P<0.001$). We also ex-

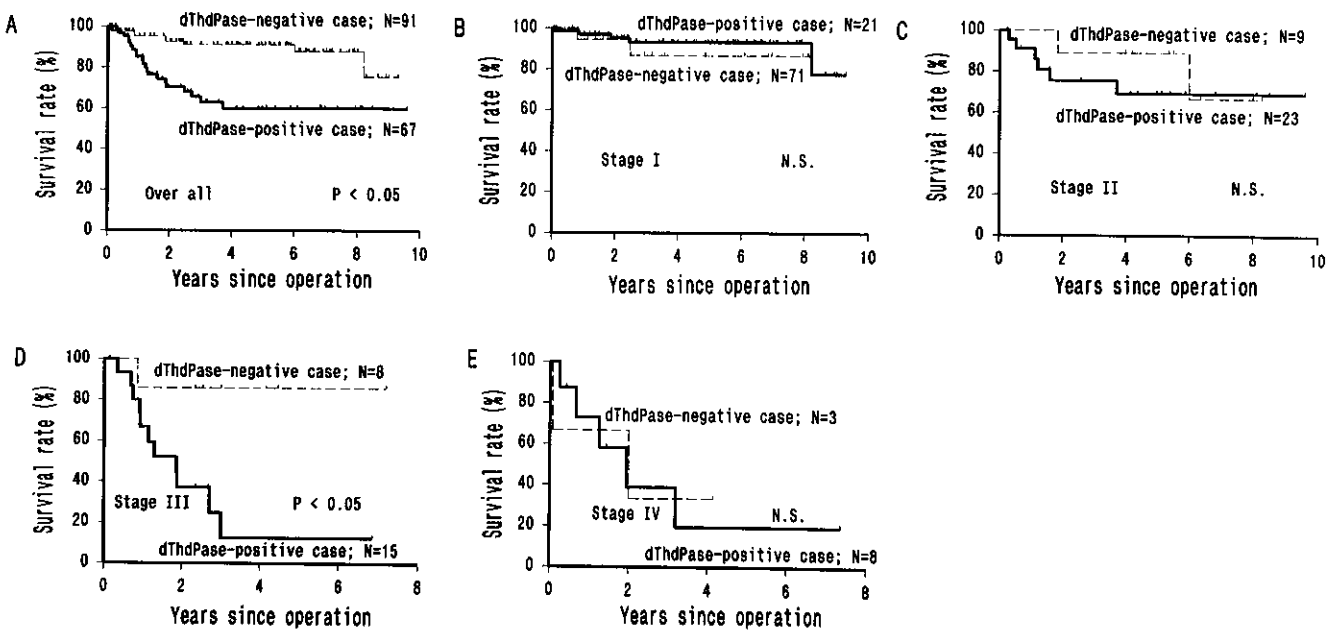

Fig. 5. Survival curve of patients with gastric carcinomas overall (A), in stage I (B), in stage II (C), in stage III (D) and in stage IV (E).

amined the correlation between these significant clinicopathologic factors and dThdPase expression by multivariate logistic analysis. Significant factors were microvessel count ($P<0.001$) and lymph node metastasis ($P=0.013$) (Table II).

Prognostic relevance The difference of overall survival between patients with dThdPase-positive carcinomas and those with negative carcinomas was significant (Fig. 5A). The prognosis of patients with dThdPase-positive carcinomas was worse than that of patients with dThdPase-negative carcinomas. The prognosis of patients with dThdPase-positive carcinomas in stage III was also worse than that of patients with dThdPase-negative carcinomas (Fig. 5D). However, there were no significant differences in survival between patients with dThdPase-positive and negative carcinomas in stages I, II, and IV (Fig. 5B, C and E). We also evaluated the prognostic significance of dThdPase by using the Cox hazard regression model, and found no significance in this study (data not shown).

DISCUSSION

Previous studies have demonstrated increased dThdPase activity in human tumors, with higher serum levels in some patients with cancer compared to healthy controls.[4, 14, 15] In addition, using rabbit antisera against dThdPase, we have found that carcinomas of the stomach, colon and ovary have higher levels of this enzyme than adjacent normal tissues.[16, 17] Our immunohistochemical study also showed that the expression of dThdPase in gastric carcinoma was higher than that in normal mucosa.

Although the role of dThdPase in tumor proliferation is unknown, we found complete sequence identity between 120 amino acids of human dThdPase and the corresponding sequence of PD-ECGF, and we also demonstrated that rPD-ECGF has dThdPase activity.[8, 9] These observations and similar reports from other laboratories[34, 35] suggest that human dThdPase is identical with PD-ECGF.[27, 28] PD-ECGF stimulated chemotaxis of endothelial cells *in vitro* and angiogenesis *in vivo*.[10] In accordance with this, we have demonstrated that dThdPase has angiogenic activity, and that its enzymatic activity is needed for the angiogenesis.[13] These results, together with the correlation between dThdPase expression and microvessel count, support the idea that dThdPase may be concerned, at least in part, in angiogenesis in gastric carcinoma, although this remains to be proven. Although the expression of dThdPase was significantly correlated with various clinicopathologic factors, such as wall invasion, lymph node metastasis, lymphatic invasion and microvessel count, by monovariate analysis, multivariate analysis demonstrated a significant correlation between immunohistochemical positivity for dThdPase in gastric carcinomas and lymph node metastasis or microvessel count. Microvessel count was more significantly correlated than lymph node metastasis.

Angiogenesis is necessary for rapid tumor growth and the vascularized tumor extends vertically into the deep tissues beneath the basement membrane. The new proliferating capillaries disrupt the basement membranes and are more penetrable by tumor cells than mature vessels.[36] During the vascular phase, tumor cells may be shed into the circulation.[37] The clinical significance of these findings has been documented in studies of invasive breast carcinomas, where microvessel density has been shown to be significantly correlated with the occurrence of metastases.[24, 26] Prognostic variables studied using a Cox hazard regression model confirmed that dThdPase expression was an independent prognostic factor in colorectal carcinoma, although Dukes stage was the best predictor of survival (data not shown). However, expression of this enzyme in gastric carcinoma was not an independent prognostic factor (data not shown). About a half of the patients with gastric carcinoma died from peritoneal recurrence, which is not related to angiogenesis. That may be the reason why the dThdPase expression in gastric carcinoma was not an independent prognostic factor.

The present study suggests that increased expression of dThdPase in gastric carcinomas is related to angiogenesis, tumor growth, invasiveness and ability to metastasize, and the prognosis of patients in stage III.

(Received September 12, 1995/Accepted November 27, 1995)

REFERENCES

1) Iltzssch, M. H., Kouni, M. H. and Cha, S. Kinetic studies of thymidine phosphorylase from mouse liver. *Biochemistry,* **24,** 6799–6807 (1985).
2) Friedkin, D. and Roberts, D. The enzymatic synthesis of nucleosides. *J. Biol. Chem.,* **207,** 245–256 (1954).
3) Krenitsky, T. A., Koszalka, G. W. and Tuttle, J. V. Purine nucleoside synthesis, an efficient method employing nucleoside phosphorylase. *Biochemistry,* **20,** 3615–3621 (1981).
4) Zimmerman, M. and Seidenberg, J. Deoxyribosyl transfer. *J. Biol. Chem.,* **230,** 2618–2621 (1964).
5) Gallo, R. C., Perry, S. and Breitman, T. R. The enzymatic mechanisms for deoxythymidine synthesis in human leukocytes. *J. Biol. Chem.,* **242,** 5059–5068 (1967).

6) Krenitsky, T. A. Pentosyl transfer mechanisms of the mammalian nucleoside phosphorylases. *J. Biol. Chem.*, **243**, 2871–2875 (1968).
7) Desgranges, C., Razaka, G. and Rabaud, H. Catabolism of thymidine in human blood platelets-purification and properties of thymidine phosphorylase. *Biochim. Biophys. Acta*, **654**, 211–218 (1981).
8) Furukawa, T., Yoshimura, A., Sumizawa, T., Haraguchi, M., Akiyama, S., Fukui, K., Ishizawa, M. and Yamada, Y. Angiogenic factor. *Nature*, **356**, 668 (1992).
9) Sumizawa, T., Furukawa, T., Haraguchi, M., Yoshimura, A., Takeyasu, A., Ishizawa, M., Yamada, Y. and Akiyama, S. Thymidine phosphorylase activity associated with platelet-derived endothelial cell growth factor. *J. Biochem.*, **114**, 9–14 (1993).
10) Haraguchi, M., Miyadera, K., Uemura, K., Sumizawa, T., Furukawa, T., Yamada, K., Akiyama, S. and Yamada, Y. Angiogenic activity of enzymes. *Nature*, **368**, 198 (1994).
11) Ishikawa, F., Miyazono, K., Hellman, U., Drexler, H., Wernstedt, C., Hagiwara, K., Usuki, K., Takaki, F., Risau, W. W. and Heldin, C. H. Identification of angiogenic activity and the cloning and expression of platelet-derived endothelial cell growth factor. *Nature*, **338**, 557–562 (1989).
12) Miyazono, K., Okabe, T., Urabe, A., Takaku, F. and Heldin, C. H. Purification and properties of an endothelial cell growth factor from human platelets. *J. Biol. Chem.*, **262**, 4098–4103 (1987).
13) Miyadera, K., Sumizawa, T., Haraguchi, M., Yoshida, H., Konstanty, W., Yamada, Y. and Akiyama, S. Role of thymidine phosphorylase activity in the angiogenic effect of platelet-derived endothelial cell growth factor/thymidine phosphorylase. *Cancer Res.*, **55**, 1687–1690 (1995).
14) Pauly, J. L., Schuller, M. G., Zelcer, A. A., Kris, T. A. and Gore, S. S. Identification and comparative analysis of thymidine phosphorylase in the plasma of healthy subjects and cancer patients: brief communication. *J. Natl. Cancer Inst.*, **58**, 1587–1590 (1977).
15) Pauly, J. L., Paolini, N. S., Ebarb, R. L. and Germain, M. J. Elevated thymidine phosphorylase activity in the plasma and fluids of tumor-bearing animals (40034). *Exp. Biol. Med.*, **157**, 262–267 (1978).
16) Yoshimura, A., Kuwazuru, Y., Furukawa, T., Yoshida, H., Yamada, K. and Akiyama, S. Purification and tissue distribution of human thymidine phosphorylase; high expression in lymphocytes, reticulocytes and tumors. *Biochim. Biophys. Acta*, **1034**, 107–113 (1990).
17) Takebayashi, Y., Yamada, K., Maruyama, I., Fujii, R., Akiyama, S. and Aikou, T. The expression of thymidine phosphorylase and thrombomodulin in colorectal carcinoma. *Cancer Lett.*, **92**, 1–7 (1995).
18) Folkman, J. and Klagsbrun, M. Angiogenic factor. *Science*, **235**, 442–447 (1987).
19) Folkman, J. What is the evidence that tumors are angiogenesis dependent? *J. Natl. Cancer Inst.*, **82**, 4–6 (1990).
20) Liotta, L., Kleinerman, J. and Saidel, G. Quantitative relationships of intravascular tumor cells, tumor vessels, and pulmonary metastases following tumor implantation. *Cancer Res.*, **34**, 997–1004 (1974).
21) Srivastava, A., Laidler, P., Hughes, L. E., Woodcock, J. and Shedden, E. J. Neovascularization in human cutaneous melanoma: a quantitative morphological and Doppler ultrasound study. *Eur. J. Cancer Clin. Oncol.*, **22**, 1205–1209 (1986).
22) Srivastava, A., Laidler, P., Davies, R. P., Horgan, K. and Hughes, L. E. The prognostic significance of tumor vascularity in intermediate-thickness (0.76–4.0 mm thick) skin melanoma: a quantitative histologic study. *Am. J. Pathol.*, **133**, 419–423 (1988).
23) Herlyn, M., Clark, W. H., Rodeck, U., Mancianti, M. L., Jambrosic, J. and Koprowski, H. Biology of tumor progression in human melanocytes. *Lab. Invest.*, **56**, 461–474 (1987).
24) Weidner, N., Semple, J. P., Welch, W. R. and Folkman, J. Tumor angiogenesis and metastasis ··· correlation in invasive breast carcinoma. *N. Engl. J. Med.*, **324**, 1–8 (1991).
25) Weidner, N., Folkman, J., Pozza, F., Bevilacqua, P., Allred, E. N. and Moore, D. H. Tumor angiogenesis: a new significant and independent prognostic indicator in early-stage breast carcinoma. *J. Natl. Cancer Inst.*, **84**, 1875–1887 (1992).
26) Horak, E. R., Leek, R., Klenk, N., Lejeune, S., Smith, K., Stuart, N., Greenall, M., Stepnieuska, K. and Harris, A. L. Angiogenesis assessed by platelet-derived cell adhesion molecular antibodies, as indicator of node metastases and survival in breast cancer. *Lancet*, **340**, 1120–1124 (1992).
27) Weinder, N., Carroll, P. R., Flax, J., Blumenfeld, W. and Folkman, J. Tumor angiogenesis correlates with metastasis in invasive prostate carcinoma. *Am. J. Pathol.*, **143**, 401–409 (1993).
28) Macchiarini, P., Fontanini, G., Hardin, M. J., Squartini, F. and Angeletti, C. A. Relation of neovascularization to metastasis of non-small-cell lung cancer. *Lancet*, **340**, 145–146 (1992).
29) Yamazaki, K., Abe, S., Takekawa, H., Sudou, N., Watanabe, N. and Ogura, S. Tumor angiogenesis in human lung adenocarcinoma. *Cancer*, **74**, 2245–2250 (1995).
30) Japanese Research Society for Gastric Cancer. The general rules for the gastric cancer study in surgery and pathology. *Jpn. J. Surg.*, **11**, 127–145 (1981).
31) Hsu, S. M., Raine, L. and Fanger, A. H. A comparative study of the peroxidase method and an avidin-biotin complex method for studying polypeptide hormones with radioimmunoassay antibody. *Am. J. Clin. Pathol.*, **75**, 734–738 (1981).
32) Kaplan, E. L. and Meier, P. Nonparametric estimation from incomplete observation. *J. Am. Stat. Assoc.*, **16**, 95–101 (1977).
33) Peto, R., Pike, M. C., Armitage, P., Breslow, N. E., Cox, D. R., Howard, S. V., Mantel, N., McPherson, K., Peto, J. and Smith, P. G. Design and analysis of randomized clinical trials requiring prolonged observation of each pa-

tient. *Br. J. Cancer,* **35,** 1–27 (1977).
34) Usuki, K., Saras, J., Waltenberger, J., Miyazono, K., Pierce, G., Thomason, A. and Heldin, C. H. Platelet-derived endothelial cell growth factor has thymidine phosphorylase activity. *Biochem. Biophys. Res. Commun.,* **184,** 1311–1316 (1992).
35) Moghaddam, A. and Bicknell, R. Expression of platelet-derived endothelial cell growth factor in *Escherichia coli* and confirmation of its thymidine phosphorylase activity. *Biochemistry,* **31,** 12141–12146 (1992).
36) Nagy, J. A., Brown, L. F., Senger, D. R., Lanir, N., Watter, L. V. D., Dvorak, A. M. and Dvorak, H. F. Pathogenesis of tumor stroma generation: a critical role for leaky blood vessels and fibrin deposition. *Biochim. Biophys. Acta,* **948,** 305–326 (1989).
37) Liotta, L. A., Saidel, G. and Kleinerman, J. The significance of hematogenous tumor cell clumps in the metastatic process. *Cancer Res.,* **36,** 889–894 (1967).