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Thymidine phosphorylase expression results in a decrease in apoptosis and increase in intratumoral microvessel density in human gastric carcinomas

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Abstract Thymidine phosphorylase (dThdPase) / platelet-derived endothelial cell growth factor (PD-ECGF) is expressed at higher levels in a variety of human carcinomas than in adjacent normal tissue. The higher expression is associated with an increase in intratumoral microvessel density (IMVD) and an unfavorable patient prognosis. This study examined the role of dThdPase in apoptosis, IMVD and p53 expression in human gastric carcinomas. dThdPase expression was noted in 12 (35.3%) of 34 early carcinomas, and in 20 (55.6%) of 36 advanced carcinomas. At least 10 areas consisting of carcinoma cells with diffuse dThdPase expression from the 32 dThdPase-positive tumors (category I), and 10 areas without dThdPase expression from the 38 negative tumors (category II) were selected from each case. For early gastric carcinoma, the mean IMVD was 88.8±19.4 in category I and 61.4±17.3 in category II carcinomas, while for advanced gastric carcinoma, the mean IMVD was 98.8±21.0 in category I and 76.0±27.1 in category II carcinomas. The mean IMVD was significantly higher in category I than in category II tumors ($P<0.05$). The mean apoptotic index (AI: percentage of apoptotic cells) was 1.95±1.30 in category I, and 3.76±1.49 in category II carcinomas for early gastric carcinoma, and 1.51±0.98 in category I and 2.14±0.66 in category II carcinomas for advanced gastric carcinoma, the value of the mean AI being significantly ($P<0.05$) higher in dThdPase-negative tumors (category II) than in the positive tumors (category I), regardless of tumor stage or histological type. There was a significant inverse correlation ($P<0.001$) between AI and IMVD. These results indicate that dThdPase expression is associated with both an increase in intratumoral microvessels and a decrease in apoptosis in human gastric carcinomas.

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Introduction

Thymidine phosphorylase (dThdPase), a member of the pyrimidine nucleoside phosphorylase (PyNPase) family, is an enzyme involved in the salvage pathway of pyrimidine nucleotide synthesis [35]. This enzyme also converts 5'-deoxy-5-fluorouridine (5'-DFUR), a prodrug of 5-fluorouracil (5-FU), to 5-FU [9, 15, 16, 38]. As a result, dThdPase has an important role in the expression of the anti-tumor activity of 5'-DFUR. Recent studies have demonstrated that dThdPase is identical to platelet-derived endothelial cell growth factor (PD-ECGF), which has been shown to possess potent angiogenic activity both in vivo and in vitro [4, 7, 12, 28].

dThdPase expression has been shown to be higher in malignant tissue than in normal tissue in a variety of human carcinomas, including bronchial, mammary, colorectal, pancreatic, and gastric carcinomas [6, 19, 24, 26, 29–32, 34, 37]. The higher expression of dThdPase has also been shown to be well correlated with intratumoral microvessel density (IMVD), a high invasive potential, tumor metastasis, and unfavorable prognosis for cancer patients [10, 17, 22, 30, 31].

Recently, Takebayashi et al. [30] have demonstrated that dThdPase expression is a prognostic factor independent of angiogenesis in human colorectal carcinomas. Similarly, Moghaddam et al. [23] reported an increase in the growth of human breast carcinomas expressing dThdPase without an increase in IMVD. These results suggest that dThdPase possesses functions other than inducing intratumoral angiogenesis that affect tumor growth. Kitazono et al. [14] examined the relationship between dThdPase expression and apoptosis using human cultured epidermoid carcinoma KB cells in vitro. They found that dThdPase, and also a degradation product of thymidine, 2-deoxy-D-ribose, conferred resistance to apoptosis induced by hypoxia in the KB cells. This

could indicate that dThdPase is involved in the intracellular apoptotic signal transduction pathway, although the precise mechanism has not been elucidated. More recently, we have reported that dThdPase expression provided an advantage for tumor growth of human colorectal carcinomas, not only by increasing the intratumoral microvessels, but also by attenuation of apoptosis, which may occur via a *p53* gene-independent pathway [21]. The role of dThdPase expression, however, has not been analyzed in human gastric carcinomas in terms of apoptosis and cell proliferation.

Here, we examine the dThdPase expression in human gastric carcinomas, comparing histological type and stage, tumor cell apoptosis, intratumoral angiogenesis, and p53 expression. Our results provide in vivo evidence that dThdPase expression is associated with not only angiogenesis, but also protection against apoptosis for gastric carcinoma cells.

Materials and methods

Tissue samples

Studies were conducted on 70 surgical specimens of gastric adenocarcinoma extracted from the files of the Department of Pathology, Faculty of Medicine, Tottori University and its related teaching hospitals. The patients, who underwent surgery, received neither chemotherapy nor radiation before the operation. Routinely processed, formalin-fixed, paraffin-embedded tissue blocks containing the principal infiltration of the tumoral mass were selected. Sections 3 µm thick were examined by light microscopy, immunohistochemistry, and the TUNEL procedure. The histological classification and tumor stage were routinely made according to the criteria of the Japanese Gastric Cancer Association [13]. In this study, we classified the tumor into two types: well-differentiated (papillary and tubular carcinomas) and poorly differentiated (poorly differentiated and signet-ring cell carcinomas). The classification corresponds to the intestinal type in the former, and diffuse type in the latter (Lauren's classification 1965).

Immunohistochemistry

Dewaxed paraffin sections were immunostained using the streptavidin–biotin peroxidase complex (SAB) method. The following primary antibodies were used: monoclonal antibodies raised against CD34 (NU-4A1; Nichirei, Japan), p53 (BP53–12, diluted 1:50; Novocastra Laboratories, Newcastle, U.K.), and dThdPase (Anti-dThdPase mouse monoclonal antibody 654-1, diluted 1:1000; Nippon Roche Research Center, Kanagawa, Japan). All the sections were heated in a 10 mM citrate buffer, pH 6.0, in a microwave oven for 15 min at 94°C. Immunoreactions were visualized with diaminobenzidine (DAB) and the sections were counterstained with 3% methyl green.

To examine the specificity of immunostaining, the primary antibody was replaced by mouse normal IgG at a 1:100 dilution and Tris-buffered saline. Control slides were invariably negative for immunostaining. We immunostained appropriate control slides of gastric carcinoma, which were previously known to show the positive immnoreactivity for CD34, p53, and dThdPase, at the same time, to obtain constant findings.

We judged samples to be positive for p53 or dThdPase when at least 10% of the cancer cells were stained.

TUNEL procedure

To detect DNA breaks in situ, terminal deoxynucleotidyl transferase(TdT)-mediated dUTP-digoxigenin nick-end labeling (TUNEL) was performed according to the method of Gavrieli et al. [5], using an Apop Tag Plus in situ apoptosis detection kit (Intergen, USA). Briefly, after deparaffinization and blocking of endogenous peroxidase with 2% hydrogen peroxidase (H_2O_2) in methanol for 30 min at room temperature, sections were incubated with 20 µg/ml proteinase K (Boehringer Mannheim/Yamanouchi, Tokyo, Japan) for 40 min at 37°C. After prehybridization treatment, the sections were incubated with terminal deoxynucleotidyl transferase (TdT), digoxigenin-11-dUTP and dATP in a moist chamber for 90 min at 37°C. Incubation with anti-digoxigenin–antibody-peroxidase for 30 min at room temperature was employed for detection of digoxigenin-11-dUTP labeling, followed by color development with a solution containing 3,3'-diaminobenzidine and H_2O_2. Methyl green was used for counterstaining.

To examine the specificity of TUNEL procedure, slides were treated with TdT buffer solution not containing TdT or digoxigenin-11-dUTP as negative controls, which were invariably negative for TUNEL signals. At the same time, the TUNEL procedure was also conducted with control slides of gastric carcinoma, which were already known to show a lot of apoptotic cells with TUNEL signals, to obtain constant findings.

Evaluation of apoptotic index and IMVD

The apoptotic index (AI: percentage of apoptotic cells) of each gastric carcinoma was obtained as the number of TUNEL-positive cancer cells per 1,000 cancer cells in the most frequently labeled area. We evaluated the mean AI and IMVD in areas which were selected using the following criteria: areas consisting of carcinoma cells with diffuse dThdPase expression from the dThdPase-positive tumors, and areas without dThdPase expression from negative tumors. For the determination of IMVD, using the CD34, at least 10 of the most highly vascularized areas within a section were selected and counted under a light microscope with 200-fold magnification, as described by Weidner et al. [36]. The average numbers were recorded as the IMVD for each case. The IMVD was counted by the two authors (M.O. and H.I.) independently without knowledge of the clinical information, and then adjusted.

Statistical analysis

The correlation between AI and IMVD was analyzed using the Spearman rank correlation coefficient. The Mann-Whitney's U-test was used for analyzing statistical correlations among IMVD, AI and p53 expression. A P-value <0.05 was considered significant.

Results

dThdPase immunoreactivity

dThdPase immunoreactivity was observed in both the nucleus and the cytoplasm of the gastric carcinoma cells, the immunoreaction being more predominant in the latter than in the former (Fig. 1a, b). The immunoreactivity was also noted in stromal lymphocytes, fibroblasts, and macrophages to varying degrees, as described previously in an analysis of colonic carcinomas [21, 26].

dThdPase expression was noted in 21 (43.8%) of the 48 well-differentiated carcinomas and in 11 (50.0%) of the 22 poorly differentiated carcinomas, the frequency

showing no significant difference (Table 1). dThdPase-positive samples were noted in 12 (35.3%) of the 34 early carcinomas, and in 20 (55.6%) of the 36 advanced carcinomas, respectively (Table 2). The frequency of dThdPase-positive cases was significantly higher in the advanced carcinomas than in the early carcinomas, regardless of the histological type (Tables 1, 2).

Table 1 Expression of dThdPase and histlogical type in gastric carcinoma

Histological type	No. of cases	No. of positive cases
Intestinal type	48	21 (43.8%)
Early	28	10 (35.7%)
Advanced	20	11 (55.0%)
Diffuse type	22	11 (50.0%)
Early	6	2 (33.3%)
Advanced	16	9 (56.3%)

dThdPase expression and IMVD

We selected at least 10 areas containing cancer cells with diffuse dThdPase expression from the 32 dThdPase-positive cases (category I), and similarly, at least 10 areas without dThdPase expression from the 38 negative cases (category II). Intratumoral microvessels were clearly demonstrated with CD34 immunostaining (Fig. 2a, b). Table 2 shows the relationship between the dThdPase expression and mean IMVDs of the gastric carcinomas. For early gastric carcinomas, the mean IMVD was 88.8±19.4 in the 12 category I carcinomas and 61.4±17.3 in the 22 category II carcinomas. Similarly, the mean IMVD was

Table 2 Relationship between TP expression, apoptotic index (*AI*), intratumoral microvessel density (*IMVD*) and p53 in human gastric carcinomas (*dThdPase* thymidine phosphorylase)

Stage/category	IMVD[a]	AI[a]	p53[b]
Early (*n*=34)			
Category I dThdPase (+) *n*=12 (35.3%)	88.8±19.4	1.95±1.30	10 (83.3%)
Category II dThdPase (–) *n*=22 (64.7%)	61.4±17.3	3.76±1.49	5 (22.7%)
	**	**	
Advanced (*n*=36)			
Category I dThdPase (+) *n*=20 (55.6%)	98.8±21.0	1.51±0.98	11 (55.0%)
Category II dThdPase (–) *n*=16 (44.4%)	76.0±27.1	2.14±0.66	8 (50.0%)
	*	*	

*$P<0.05$ versus control;
**$P<0.01$ versus control
[a] Values are expressed as mean±SE
[b] Number of positive cases (% in parentheses)

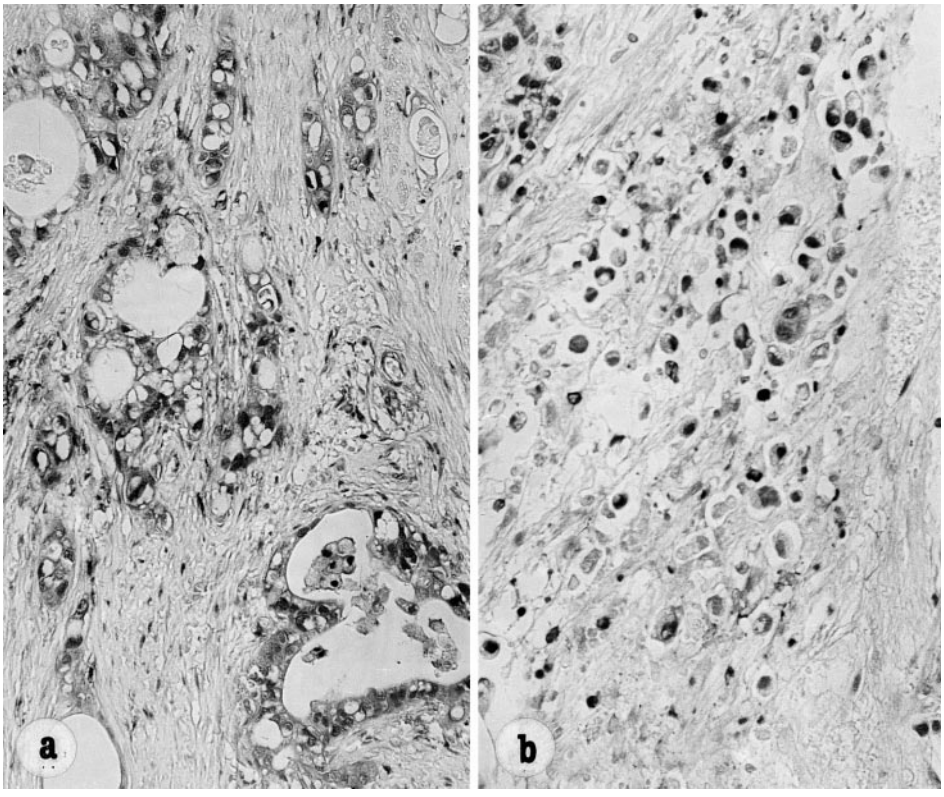

Fig. 1a, b Thymidine phosphorylase (dThdPase) expression in human gastric carcinoma. The expression is noted in both nucleus and cytoplasm of carcinoma cells, as well as stromal lymphoid cells and fibroblasts. Immunostaining for dThdPase, ×180
a Well-differentiated adenocarcinoma, **b** poorly differentiated adenocarcinoma

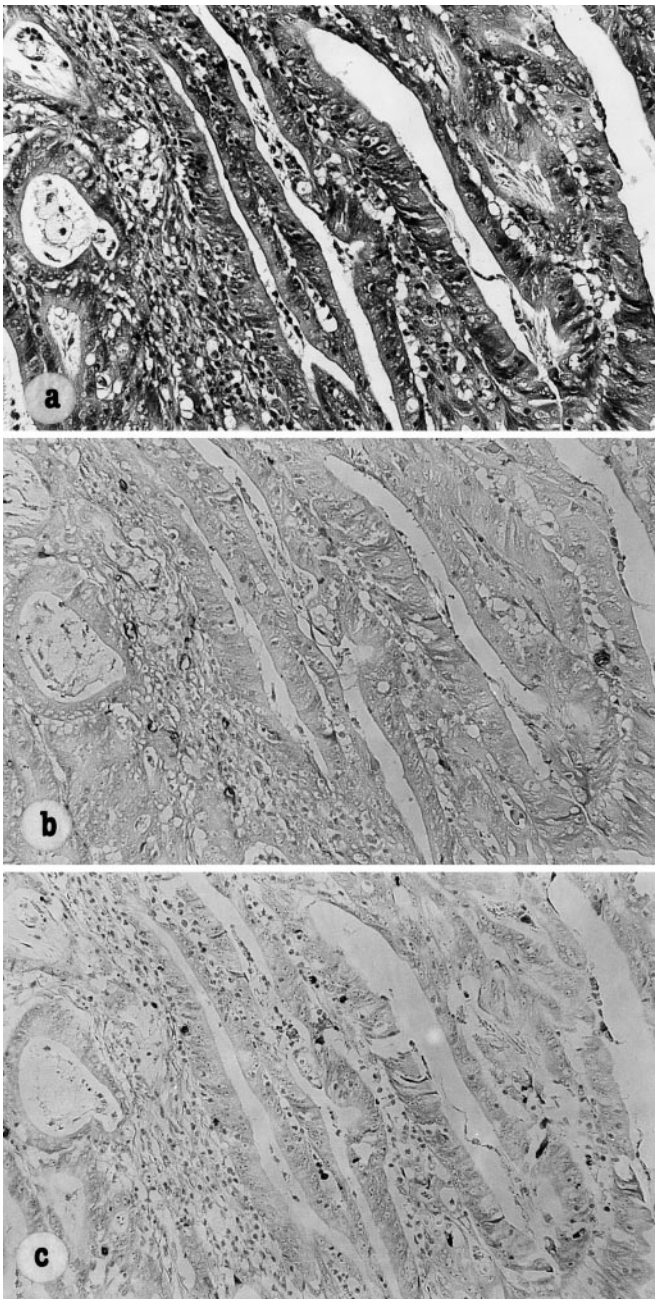

Fig. 2 a Intratumoral microvessels (*IMV*) and TUNEL-positive tumor cells in dThdPase-negative gastric carcinoma. H&E, ×170 **b** CD-34 immunoreactivity demonstrated a few intratumoral microvessels. ×170 **c** Scattered TUNEL-signal-positive tumor cells are present. ×170

98.8±21.0 in the 20 category I carcinomas and 76.0±27.1 in the 16 category II carcinomas for advanced gastric carcinomas. The mean IMVD was significantly (P<0.05) higher in the dThdPase-positive carcinomas (category I) than in the dThdPase-negative carcinomas (category II). However, there was no statistical difference in the mean IMVD in each category: 88.8 versus 98.8 in category I, and 61.4 versus 76.0 in category II. Thus, the mean IMVD was associated with dThdPase expression, but not with the tumor stage in either category.

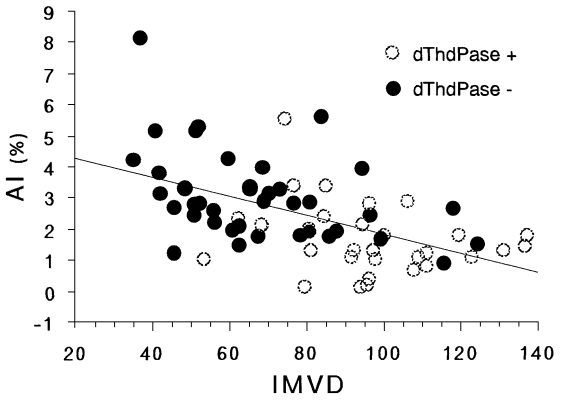

Fig. 3 Spearman rank correlation coefficient test for correlation between apoptotic index (*AI*) and intratumoral microvessel density (*IMVD*). For all cases, r=0.19, P<0.001

Correlation between AI and IMVD, and p53 expression

Next, we analyzed the apoptotic indices in the gastric carcinomas (Table 2, Fig. 2a, c). The mean AI was 1.95±1.30 in the 12 category I carcinomas, and 3.76± 1.49 in the 22 category II carcinomas in the early stages, and 1.51±0.98 in category I (n=20) and 2.14±0.66 in category II carcinomas (n=16) in the advanced stages. The mean AI was significantly lower in the dThdPase-positive carcinomas than in the negative carcinomas (P<0.05), regardless of the tumor stage.

Regression analysis of the Spearman rank correlation coefficient, on plots of AI versus IMVD on a per case basis, showed a significant inverse correlation between AI and IMVD (r=0.19, P<0.001; Fig. 3).

Nuclear P53 expression was noted in 10 (83.3%) of 12 category I carcinomas, and 5 (22.7%) of 22 category II carcinomas in the early stages, and 11 (55.0%) in category I (n=20) and 8 (50.0%) in category II carcinomas (n=16) in the advanced stages. The frequency was significantly higher in the dThdPase-positive early carcinomas than in the negative early carcinomas (P<0.05). On the other hand, there was no correlation between dThdPase and p53 expression in the advanced carcinomas.

Discussion

In this study, we clearly demonstrated that dThdPase is immunohistochemically expressed in both the cancer cells and the stromal cells of human gastric carcinomas. The expression was shown to be associated with IMVD. Although the precise mechanism of angiogenesis by dThdPase remains unclear, previous reports have demonstrated that the expression of dThdPase is significantly associated with IMVD [10, 30, 33]. In this study, higher IMVD was clearly demonstrated in dThdPase-positive gastric carcinomas than in negative carcinomas, regardless of the tumor stage or histological type. These results suggest that the expression of dThdPase has a crucial role in intratumoral angiogenesis, which occurs in the early phase of gastric carcinoma.

Several reports are now available on the clinical significance of dThdPase in human gastric carcinomas. Takebayshi et al. [31] demonstrated a significantly worse prognosis in gastric carcinoma patients with dThdPase expression than in those without it. They confirmed positive significance only in 23 patients of stage III, but not in stages I, II and IV. Maeda et al. [20] found that the frequency of hepatic metastasis was significantly higher in patients with dThdPase-positive carcinomas than in those with negative carcinomas. On the other hand, they failed to demonstrate any significant correlation between dThdPase expression and patient prognosis. Thus, the clinicopathological significance of dThdPase expression is even now controversial in human gastric carcinomas.

As alluded to in the introductory section, recent studies have demonstrated that dThdPase expression is associated with the apoptosis-resistant nature of tumor cells both in vivo and in vitro. We also confirmed a decrease in apoptotic cell death in human colorectal carcinomas with diffuse dThdPase expression [21]. Similar results were confirmed in human gastric carcinomas in this study. Lu et al. [18] reported the relationship between spontaneous apoptosis and IMVD in human gastric carcinomas. Our data are consistent with their report, in which the incidence of apoptosis was significantly influenced by the extent of neovascularization, suggesting that tumor angiogenesis contributes to the reduction in apoptosis in tumor cells. It is also suggested that the expression of dThdPase is associated with the apoptotic mechanism in carcinogenesis. It cannot be denied that dThdPase could be directly involved in intracellular apoptotic signal transduction, although the precise mechanism has not yet been elucidated. In any case, dThdPase expression provides an advantage for tumor growth in human gastric carcinomas, not only by forming a favorable, highly vascular intratumoral microenvironment, but also by allowing cells to escape the apoptotic mechanism.

Apoptosis is regulated by a variety of oncogenes and suppressor genes. Among the alterations, the *p53* suppressor gene plays a crucial part in the induction of apoptosis. The product of the wild-type *p53* gene has been shown to be required for induction of the apoptotic pathway triggered by oncogenous activation and cytotoxic genes [1, 27]. The product may sensitize damaged cells to apoptosis, acting to prevent the propagation of transforming mutations. The expression of nuclear p53 almost matches that of the mutant type [3]. We previously reported that the expression of a mutated *p53* gene attenuated apoptotic cell death in gastric cancer in vivo [11]. In the present study, p53 expression seemingly correlated with dThdPase expression and lower AI in early gastric carcinomas, but not in advanced carcinomas. This may be partly due to the small number of dThdPase-positive early gastric carcinomas. In fact, we failed to demonstrate the correlation between the p53 protein and dThdPase expression in another study analyzing gastric carcinomas [25], and we were also not able to confirm any correlation in breast and colonic carcinomas. Thus, attenuation of apoptotic cell death seems to occur via a *p53* gene-independent pathway in gastric carcinomas with dThdPase expression.

The precise mechanism of the apoptosis resistance of dThdPase-expressing carcinoma cells awaits further clarification. Recently, it has been reported that dThdPase expression is regulated by various cytokines and growth factors, including tumor necrosis factor-α, interleukin-1, interferon-γ and basic fibroblast growth factor (bFGF) [2, 8]. Thus, dThdPase expression is considered to be regulated by various factors via the autocrine and paracrine pathways.

In summary, we have clearly demonstrated by way of in vivo evidence that dThdPase expression attenuates apoptotic cell death and increases IMVD, which provides a preferential advantage for tumor cell proliferation in human gastric carcinomas. Further study may provide information valuable for an emerging concept; antiangiogenesis therapy.

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