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Clinicopathologic and Prognostic Significance of an Angiogenic Factor, Thymidine Phosphorylase, in Human Colorectal Carcinoma

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Background: Platelet-derived endothelial cell growth factor (PD-ECGF) is known to promote the development of new blood vessels, which are fundamental to tumor growth and metastasis. We previously found that thymidine phosphorylase (dThdPase) and PD-ECGF are the same protein.

Purpose: We retrospectively examined the expression of dThdPase in primary colorectal carcinomas, its association with angiogenesis and clinicopathologic findings, and its prognostic value.

Methods: Tissues were obtained from the tumors of 163 patients whose colorectal carcinomas were completely removed by surgery. Microvessels assessed by immunostaining endothelial cells for factor VIII were counted on a 400x field in the most active areas of neovascularization within the tumor. We purified the monoclonal antibody against dThdPase and studied the expression of dThdPase in the same serial sections used for the detection of factor VIII. Those who carried out microvessel counting and dThdPase expression assessment had no knowledge of clinicopathologic findings. The significance of dThdPase in the prognosis of patients with colorectal carcinomas was also examined in the survival analysis of mortality follow-up data covering the period between 1984 through 1991. Reported P values are from two-sided tests of statistical significance.

Results: The mean microvessel count (+ standard deviation) in dThdPase-positive colorectal carcinoma specimens (17.5 ± 7.2) was higher (P<.001) than that in dThdPase-negative carcinoma specimens (9.3 ± 5.5). The dThdPase positivity was in accordance with the microvessel count. dThdPase positivity showed highly significant statistical associations with tumor size, extent of invasion, lymph node metastasis, lymphatic invasion, and venous invasion. Cox regression analysis revealed that dThdPase expression was prognostic for poor disease outcome after adjustment for Dukes' stage and microvessel count.

Conclusions: These findings suggest that higher levels of dThdPase expression in colorectal carcinomas are associated with more extensive angiogenesis, poor clinical and laboratory findings, and unfavorable clinical outcome. Implications: Inhibition of dThdPase in human colorectal carcinomas might improve prognosis for some patients. [J Natl Cancer Inst 1996; 88:1110-7]

Thymidine phosphorylase (dThdPase; Enzyme Commission No. 2.4.2.4) catalyzes the reversible phosphorolysis of thymidine, deoxyuridine, and their analogues to their respective bases and 2-deoxyribose-1-phosphate (1-3). dThdPase also catalyzes the deoxyribosyl transfer from one deoxynucleoside to another base to form a second deoxynucleoside (4-6). In mammals, dThdPase consists of two identical subunits, and the molecular weight of each subunit is about 55 kd (7). We have previously shown that dThdPase is identical to platelet-derived endothelial cell growth factor (PD-ECGF) (8-10). This protein stimulates chemotaxis and [3H]thymidine incorporation of endothelial cells in vitro and has an angiogenic activity in vivo (10-12). It does not have signal sequence, and thus it might not be a secretory protein (13). Recently, we have demonstrated that...
the enzymatic activity of dThdPase is essential to its angiogenic effects (10,14). When compared with adjacent normal tissues, dThdPase activity has been reported to increase in a variety of malignant tumors (4,15-18). The expression of dThdPase has been reported to be significantly higher in colorectal carcinomas than in normal colorectal tissues and adenomas, and its activity has been correlated with the expression of thrombomodulin, an endothelial cell marker (18).

Experimental evidence has shown that tumor growth is dependent on angiogenesis (19,20). When tumors reach the size of a few millimeters in diameter, capillaries penetrate, allowing for rapid tumor 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 metastasis (21-24). Recently, the expression of vascular endothelial cell growth factor, another angiogenic factor, and its receptor in colorectal carcinomas, was found to be associated with the invasiveness and progression of tumors (25).

We retrospectively examined the expression of dThdPase in primary colorectal carcinomas, its association with angiogenesis and clinicopathologic findings, and its prognostic value.

Patients and Methods

Patients and Tumor Samples

We examined 163 patients (107 males and 56 females) whose tumors were completely removed surgically in the First Department of Surgery of Kagoshima University Hospital, Japan, between January 1984 through December 1991. None had received prior chemotherapy or irradiation. The patients included in this study had no other cancers. The clinicopathologic characteristics of 163 patients with colorectal adenocarcinomas investigated in this study are summarized in Table 1. The average age at surgery was 61 years (range, 22-91 years). Patients with Dukes' C stage disease received postoperative therapy; however, there was no difference in outcome among the various treatment modalities. Follow-up for the patients included in the survival analysis was updated in January 1995 (median follow-up was 92 months [range, 2-128 months]). The immunohistochemical evaluations were completed in December 1994. At that time, 33 patients had died of colorectal carcinoma, 109 were alive, and 21 had died of other diseases. Tumors were classified histopathologically as well-differentiated, moderately differentiated, or poorly differentiated adenocarcinomas according to the World Health Organization criteria proposed by Jass and Sobin in 1989. When more than 50% of the tumor volume was mucin, the tumor was defined as mucinous carcinoma (26). All routine sections were carefully investigated to identify venous or lymphatic invasion. Histopathologic diagnosis was made routinely at the Department of Pathology of Kagoshima University Hospital. The largest diameter of the tumor was defined as the tumor size. The extent of tumor invasion/metastasis was based on the Astler and Collier (27) modification of Dukes' classification system. Dukes' A (A1 + A2) cases were those in which the growth was confined to the submucosa of the colorectal wall. In Dukes' B (B1 + B2) cases, the growth spread by direct continuity into the extracolorectal tissues, but the lymph nodes were free from metastases. Dukes' C (C1 + C2) cases were those in which lymph node metastases were found. In this study, there was one patient with lymph node metastasis whose depth of invasion was confined to the submucosa. Tumor specimens were collected after obtaining informed consent in accordance with institutional guidelines. The deepest invasive sites were selected for immunohistochemistry of factor VIII and dThdPase.

Preparation of Monoclonal Antibody Against dThdPase

Monoclonal antibodies were raised against a glutathione S-transferase (GST) dThdPase fusion product containing 244 amino acids of GST located in the first 250 residues of dThdPase. A 0.8-kilobase fragment encoding the 5' half of the coding sequence was isolated by Xma I digestion of plasmid pPL8 carrying the full-length PD-ECGF complementary DNA (cDNA) (supplied by Drs. K. Miyazono and C. H. Heldin, Ludwig Institute for Cancer Research, Uppsala, Sweden). Following insertion into pGEX-2T (Pharmacia LKB Biotechnology AB, Uppsala, Sweden) and transfection, cultures of Escherichia coli were incubated with isopropyl-β-D-thiogalactopyranoside for 4 hours at 37°C, centrifuged for 10 minutes at 2000g at 4°C, and lysed with 2× sample buffer (50 mmol/L Tris-HCl [pH 6.8] containing dodecyl sulfate [SDS], 20% glycerol, 10% 2-mercaptoethanol, and 0.002% bromophenol blue). The lysate was applied to a preparative electrophoresis apparatus (Nihon Eido, Tokyo, NA-1800 type) and separated. Each 0.3-mL fraction was collected and examined by polyacrylamide gel electrophoresis with Coomassie blue staining. The five fractions containing the 53-kd GST fusion product were collected (total protein, 6.4 mg) and used for immunizations.

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 96% ethanol. Endogenous peroxidase was blocked by immersing the slides in 0.3% hydrogen peroxide in absolute methanol for 20 minutes at room temperature. After washing three times with PBS for 5 minutes, the sections were then blocked by treating with PBS containing 1% bovine serum albumin for 20 minutes at room temperature. The blocked sections were incubated at 4°C overnight with monoclonal antibody against dThdPase diluted 500-fold with PBS, then incubated for 30 minutes with biotinylated anti-mouse immunoglobulin G (IgG) diluted 100-fold with PBS at room temperature (28). After washing three times in PBS for 15 minutes, the sections were incubated for 30 minutes with avidin and biotinylated horseradish peroxidase complex diluted 100-fold with PBS. The sections were then washed three times in PBS for 15 minutes, and the immune complex was visualized by incubating the sections with the 0.5% 3,3'-diaminobenzidine and 0.03% (vol/vol) H2O2 in PBS for 7 minutes. The sections were counterstained with hematoxylin and mounted. Staining with an irrelevant mouse IgG was routinely performed as a negative control and a normal liver section was used as a positive control, since the Kupffer cells expressed the high level of dThdPase (29,30). The serial sections were also incubated with rabbit antiserum antibody against human von Willebrand factor (Dako Polyclonal, Dako Corp., 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. Other procedures were the same as dThdPase immunohistochemical staining.

For microscopic analysis, we examined 200 cells, including cancer cells or normal mucosal cells, to determine whether the cells were positive for dThdPase. We decided that the specimens should be regarded as dThdPase negative when fewer than 5% of these 200 cells were stained and positive when more than 5% of them were stained (Fig. 1).

The areas with the highest number of discrete microvessels expressing factor VIII were examined at higher power (400×) to obtain accurate microvessel counts (Fig. 2, D). The evaluation of dThdPase expression and microvessel counts was done without knowledge of the patients' clinicopathologic factors and was performed by two investigators simultaneously (Y. Takebayashi and K. Miyadera).

Statistical Analysis

Demographic and clinicopathologic characteristics were compared between patients with dThdPase-positive and -negative tumors with the use of the chi-squared or Student's t tests, and correlation between microvessel count and proportion of dThdPase-positive carcinoma cells was evaluated by Spearman test and linear regression analysis (31). We used the Kaplan-Meier method to estimate survival rates and the generalized Wilcoxon test to compare the two groups for difference in survival rates (32). The Cox proportional hazards model was used in the multivariate survival analysis (33). Maximum likelihood parameter estimates and likelihood ratio statistics (LRS) in the Cox proportional hazards models were obtained with the use of a statistical package, EPICURE (34). We calculated Wald-type confidence intervals. Tests for statistical interaction were conducted by including a cross-product term of the two variables of interest in a model. All P values presented were two-sided.
Results

dThdPase Expression in Colorectal Carcinoma or Normal Sections

Most normal colorectal mucosal cells were not stained with the anti-dThdPase antibody (Fig. 2, A). In contrast, the cytoplasm of many colorectal carcinoma cells stained strongly, as did stromal cells that might be macrophages or fibroblasts (Fig. 2, B). A statistically significant (P<.001) difference in the expression of dThdPase was expressed in 70 (42.9%) of 163 colorectal carcinomas when compared with 11 (6.7%) of 163 normal colorectal tissues.
Relationship Between the Expression of dThdPase and Microvessel Count

As shown in Fig. 3, the percentage of dThdPase-positive cells increased with increasing microvessel count in the colorectum, colon, and rectum. In tumors with more than 20 microvessels per 400x field, 84.6% of the samples were dThdPase positive. Fig. 4 shows that the mean microvessel count (± standard deviation) in dThdPase-positive colorectal carcinomas (17.5 ± 7.2) was higher than in dThdPase-negative colorectal tumors (9.3 ± 5.5) (P<.001). Similar results were obtained when colon and rectal carcinomas were analyzed separately. The mean microvessel counts for dThdPase-positive and -negative tumors were 16.2 ± 6.8 and 10.0 ± 6.1, respectively, for the colon (P<.001) and 18.5 ± 7.5 and 8.7 ± 4.8, respectively, for the rectum (P<.001).

Relationship Between Pathologic Findings, Clinical Outcome, and dThdPase Expression

Fig. 4 and Table 1 summarize the relationship between clinical or pathologic features and dThdPase expression in colorectal carcinomas. However, no significant association was found between dThdPase expression and age (Fig. 4), sex, tumor location, or histologic type (Table 1). dThdPase positivity was correlated with depth of invasion, lymph node metastasis, lymphatic invasion, venous invasion, and Dukes' stage. Fig. 4 shows that the average size (± standard deviation) of dThdPase-positive carcinomas (59.1 ± 27.0 mm) was larger than that of dThdPase-negative carcinomas (41.1 ± 28.5 mm) (P<.001).

Prognostic Relevance of dThdPase Expression

Kaplan–Meier product limit estimates of overall survival were plotted in Fig. 5. Patients with dThdPase-positive carcinomas had a significantly (P<.001) poorer survival than those with negative tumors. To determine whether dThdPase positivity was a prognostic factor independent of Dukes' stage, an established prognostic factor, and microvessel count, we conducted a survival analysis with the use of the Cox proportional hazards models (Table 2). Although it was unlikely that the other clinicopathologic variables shown in Table 2 affected patient survival, we included them in our analysis to confirm the

![Graphs showing the relationship between microvessel count and dThdPase expression.](image-url)
absence of their associations with survival after adjustment for Dukes' stage. Dukes' stage was an independent prognostic factor from the analysis using Model 0. None of the other variables included in this model was a statistically significant prognostic factor. The analysis using Model 1 showed that Dukes' stage and microvessel count were statistically significant prognostic factors independent of covariables included in the model. The analysis using Model 2, which included dThdPase positivity in addition to all of the covariables in Model 1, showed that dThdPase positivity was a prognostic factor after adjustment for Dukes' stage and microvessel count by multivariate survival analysis. To examine whether the hazard ratio for dThdPase positivity is different between the colon and rectum, the cross-product term of the two variables, representing tumor location and dThdPase positivity, was included in Model 2. The LRS for the interaction term was 0.7 (degrees of freedom [df] = 1), indicating that the effect of dThdPase positivity on overall survival was not statistically significantly different between patients with colon and rectal carcinomas ($P = .417$). We also examined whether the hazard ratio for dThdPase positivity was different between Dukes' stages. The LRS for the interaction term between dThdPase positivity and Dukes' stage was 1.0 (df = 1), indicating that there was no evidence of difference in the prognostic effect of dThdPase by Dukes' stages ($P = .318$).

**Discussion**

Previous studies ($4,15,16,30$) have demonstrated that some patients with cancer have increased serum levels of dThdPase when compared with healthy controls. With the use of rabbit antisera against dThdPase, we also found that carcinomas of the stomach, colon, and ovary have higher levels than the adjacent normal tissues ($17$). The present retrospective study confirmed increased dThdPase expression in primary colorectal carcinomas.

Although the role of dThdPase in tumor proliferation is unknown, the results in this study are consistent with the role of this enzyme in angiogenesis. In a study ($8$), we found complete sequence identity between 120 amino acids of human dThdPase and the sequence of PD-ECGF. Recombinant PD-ECGF has dThdPase activity. When COS cells were transfected with full-length PD-ECGF cDNA, the PD-ECGF expressed had dThdPase activity ($9$). These observations and similar reports from other laboratories ($35,36$) suggest that human dThdPase is identical to PD-ECGF. A previous study ($11$) has demonstrated that PD-ECGF stimulates chemotaxis of endothelial cells in vitro and angiogenesis in vivo. In accordance with this, we have demonstrated that dThdPase has angiogenic activity and that its enzymatic activity is needed for the angiogenesis ($10,14$). Interestingly, the expression of thrombomodulin, an endothelial cell marker, is significantly correlated with dThdPase activity in human colorectal carcinomas ($18$). These results, together with the correlation between dThdPase expression and microvessel count observed in this study, strongly suggest this enzyme to be an important factor in angiogenesis in human colorectal carcinomas.

Fox et al. ($29$) and Takebayashi et al. ($30$) separately reported that stromal cells (macrophages, tumor-infiltrating lymphocytes,
but the lymph nodes are free from metastases; and C = those patients in whom the growth has spread by direct continuity into the extracolorectal tissues, Dukes' stage.

Venous invasion

Lymphatic invasion

Lymph node metastasis

Depth of invasion

Histopathologic type

Tumor location

Sex

No. of patients

Variables

Table 1. Relationship between clinicopathologic features and thymidine phosphorylase (dThdPase) expression in colorectal carcinomas (n = 163)

<table>
<thead>
<tr>
<th>Variables</th>
<th>dThdPase negative (%)</th>
<th>dThdPase positive (%)</th>
<th>P*</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. of patients</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sex</td>
<td></td>
<td></td>
<td>.85</td>
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<tr>
<td>Male</td>
<td>60 (56.1)</td>
<td>47 (43.9)</td>
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</tr>
<tr>
<td>Female</td>
<td>33 (58.9)</td>
<td>23 (41.1)</td>
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<tr>
<td>Tumor location</td>
<td></td>
<td></td>
<td>.79</td>
</tr>
<tr>
<td>Colon</td>
<td>37 (63.8)</td>
<td>21 (36.2)</td>
<td></td>
</tr>
<tr>
<td>Rectum</td>
<td>56 (53.3)</td>
<td>49 (46.7)</td>
<td></td>
</tr>
<tr>
<td>Histopathologic type</td>
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</tr>
<tr>
<td>Well differentiated</td>
<td>63 (57.3)</td>
<td>47 (42.7)</td>
<td></td>
</tr>
<tr>
<td>Moderately differentiated</td>
<td>25 (58.1)</td>
<td>18 (41.9)</td>
<td></td>
</tr>
<tr>
<td>Poorly differentiated</td>
<td>0 (0)</td>
<td>4 (100.0)</td>
<td></td>
</tr>
<tr>
<td>Mucinous</td>
<td>5 (83.3)</td>
<td>1 (16.7)</td>
<td></td>
</tr>
<tr>
<td>Depth of invasion</td>
<td></td>
<td></td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Mucosa/submucosa</td>
<td>31 (91.2)</td>
<td>3 (8.8)</td>
<td></td>
</tr>
<tr>
<td>Muscle layer</td>
<td>17 (60.7)</td>
<td>11 (39.3)</td>
<td></td>
</tr>
<tr>
<td>Subserosa/serosa exposure</td>
<td>36 (43.4)</td>
<td>47 (56.6)</td>
<td></td>
</tr>
<tr>
<td>Invasion to adjacent organ</td>
<td>9 (50.0)</td>
<td>9 (50.0)</td>
<td></td>
</tr>
<tr>
<td>Lymph node metastasis</td>
<td></td>
<td></td>
<td>&lt;.001</td>
</tr>
<tr>
<td>Absent</td>
<td>72 (68.6)</td>
<td>33 (31.4)</td>
<td></td>
</tr>
<tr>
<td>Present</td>
<td>21 (36.2)</td>
<td>37 (63.8)</td>
<td></td>
</tr>
<tr>
<td>Lymphatic invasion</td>
<td></td>
<td></td>
<td>.004</td>
</tr>
<tr>
<td>Absent</td>
<td>57 (71.3)</td>
<td>23 (28.7)</td>
<td></td>
</tr>
<tr>
<td>Present</td>
<td>36 (43.4)</td>
<td>47 (56.6)</td>
<td></td>
</tr>
<tr>
<td>Venous invasion</td>
<td></td>
<td></td>
<td>.013</td>
</tr>
<tr>
<td>Absent</td>
<td>81 (62.3)</td>
<td>49 (37.7)</td>
<td></td>
</tr>
<tr>
<td>Present</td>
<td>12 (36.4)</td>
<td>21 (63.6)</td>
<td></td>
</tr>
<tr>
<td>Dukes' stage†</td>
<td></td>
<td></td>
<td>&lt;.001</td>
</tr>
<tr>
<td>A</td>
<td>30 (90.9)</td>
<td>3 (9.1)</td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>42 (58.3)</td>
<td>30 (41.7)</td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>21 (36.2)</td>
<td>37 (63.8)</td>
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</tbody>
</table>

*P values were obtained from the chi-squared test (two-sided).
†Dukes' stage was categorized as follows: A = those patients in whom the growth is confined to the submucosa of the colorectal wall; B = those patients in whom the growth has spread by direct continuity into the extracolorectal tissues, but the lymph nodes are free from metastases; and C = those patients in whom lymph node metastases are found (27).

Fig. 5. Kaplan-Meier survival curves of patients with colorectal carcinoma. Comparison of survival curves for patients whose tumors stained positive for thymidine phosphorylase (dThdPase) with those patients whose tumors were classified as negative for dThdPase expression. Curves present the results for all patients. The 95% confidence intervals (CIs) on the curves for dThdPase-positive cases at 1, 3, and 4 years were 0.97-1.00, 0.91-1.00, and 0.91-1.00, respectively. The 95% CIs on the curves for dThdPase-negative cases at 1, 3, and 4 years were 0.88-0.99, 0.56-0.79, and 0.41-0.67, respectively. Tick marks represent observations.
dThdPase may have other effects concerned with the progression of colorectal carcinomas besides angiogenic activity. In summary, we have examined dThdPase expression in colorectal carcinomas with the use of a monoclonal antibody. Expression of dThdPase was associated with tumor size, lymph node metastasis, depth of invasion, lymphatic invasion, and venous invasion through the correlation with microvessel count. dThdPase expression was, however, a prognostic factor after adjustment for Dukes’ stage and microvessel count. Although additional studies are needed to clarify the mechanism of dThdPase-mediated angiogenesis, invasiveness, and ability to metastasize, this study and previous studies (10-14) suggest that inhibitors of this enzyme suppress the growth of dThdPase-expressing tumors and are valuable in the therapy for patients with colorectal carcinomas.

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Notes

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