

• COLORECTAL CANCER •

Expression of thymidine phosphorylase by macrophages in colorectal cancer tissues

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Abstract

AIM: To detect the thymidine phosphorylase (dThdPase) expression in human colorectal cancer tissues and cells.

METHODS: Forty specimens resected from patients with colorectal cancer were immunohistochemically stained by 654-1, anti-dThdPase monoclonal antibody, PG-M1, anti-macrophage marker CD68 monoclonal antibody. Morphometrical analysis and positive cell counting were performed. In 27 of 40 specimens, dThdPase activity was also assayed by HPLC. Otherwise, the dThdPase level was measured by ELISA in 6 colorectal cancer cell lines, LS174T, Clone A, Colo320, CX-1, Lovo, and MIP101, as well as in 2 macrophage-like cell lines, THP-1 and U937.

RESULTS: dThdPase activity was significantly increased in cancer tissues compared with adjacent normal tissue ($P < 0.01$). In immunohistochemical analysis, it was confirmed that most cells expressed dThdPase were the stromal cells surrounding cancer nests or along the invasive margin of cancer. Based on their morphometrical characteristics, we found that most of them were tumor-associated macrophages (TAMs). The number of dThdPase-positive stromal cells was significantly correlated with the number of CD68-positive macrophages ($r = 0.76$, $P < 0.0001$). By ELISA, 18.2 unit/mg and 19.3 unit/mg of dThdPase protein were detected in THP-1 and U937, but only little was detected in 6 colorectal cancer cell lines.

CONCLUSION: The present data suggest that dThdPase expression is seldom detected in colorectal carcinoma cells. TAM is the most important source of dThdPase in colorectal cancer tissues.

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INTRODUCTION

Thymidine phosphorylase (dThdPase) is an enzyme involved

in pyrimidine nucleoside metabolism. It could catalyze the reversible phosphorolysis of thymidine, deoxyuridine and their analogues to their bases and 2-deoxyribose-1-phosphate^[1,2]. 5'-deoxy-5-fluorouridine (5'-DFUR), a prodrug of 5-fluorouracil (5-FU), must be activated by dThdPase in cancer tissues and converted into 5-FU, resulting in induction of the anticancer activity with few side effects in normal tissues^[3]. Previous studies have demonstrated that dThdPase is identical to platelet-derived endothelial cell growth factor (PD-ECGF), which is an endothelial cell mitogen^[4,5]. dThdPase/PD-ECGF could stimulate chemotaxis of endothelial cells *in vitro* and possess angiogenic activity *in vivo*^[5-8], however, the mechanisms by which dThdPase/PD-ECGF contributes to angiogenesis are still unclear.

Studies have reported that certain solid tumors including gastric and breast cancers could express elevated levels of dThdPase as compared with normal surrounding tissues^[9-12]. Thus it is reasonable that these tumors are susceptible to 5'-DFUR. In colorectal cancer, it is controversial whether cancer cells or stromal cells are responsible for the increased dThdPase levels. Some immunohistochemical studies reported that cancer cells had dThdPase expression^[12,13], while others believed that most cells expressing dThdPase were stromal cells, especially tumor-associated macrophages (TAMs), and lymphocytes^[14,15]. Therefore, it is uncertain how 5'-DFUR exerts its anticancer activity in colorectal cancer.

As we know, dThdPase plays an important role in cell proliferation, tumor angiogenesis, and in chemotherapy with 5'-DFUR. Here we focused on the expression of dThdPase in CD68-positive TAMs in colorectal cancer tissue with immunohistochemistry and an analysis of dThdPase level in 6 colorectal cancer cell lines by ELISA.

MATERIALS AND METHODS

Tissue preparation

Resected specimens from 40 patients with colorectal cancer in the First Department of Surgery, Tohoku University School of Medicine (Sendai, Japan) were used for immunohistochemical staining. In the 40 cases, 14 were well-differentiated adenocarcinomas, 25 were moderately differentiated carcinomas, and only 1 was poorly differentiated carcinoma.

Primary antibodies

654-1, a mouse monoclonal antibody that recognizes human dThdPase, was kindly provided by Nippon Roche Research Center (Kamakura, Japan). PG-M1, an anti-macrophage marker CD68 monoclonal antibody, was purchased from DAKO (Glostrup, Denmark). Non-specific mouse IgG1 (East Acres Biologicals, Southbridge, USA) was used as a control antibody.

Cell lines

Six human colorectal cancer cell lines, LS174T, Clone A, Colo320, CX-1, Lovo, and MIP101, and two human macrophage-like cell lines, THP-1 and U937, were used in this study. LS174T was obtained from the American Type Culture Collection (Rockville, MD). Clone A, CX-1, and MIP101 were provided by Dr. JM Jessup (University of Texas,

SA, USA). Colo320, Lovo, and THP-1, a human monocytic leukemia cell line, were purchased from Riken Cell Bank. U937, a human histiocytic lymphoma cell line, was obtained from Institute of Development, Aging and Cancer, Tohoku University (Sendai, Japan). THP-1 and U937 are suspension cell lines induced by phorbol-12-myristate-13-acetate (PMA). PMA-induced THP-1 and U937 were used in various experiments as macrophage-like cells because these cells could express many of normal macrophage characteristics including phagocytosis and adherence^[16-18]. All cell lines were cultured in RPMI1640 medium supplemented with 10% fetal calf serum (FCS) and 1% L-glutamine.

dThdPase activity assay

In 40 colorectal carcinoma specimens, 27 were frozen immediately at -80°C after removal by surgery. Then they were assayed with high performance liquid chromatography (HPLC) to detect dThdPase activity by Nippon Roche Research Center^[19].

Immunohistochemical staining

Streptavidin-biotin complex (SABC) method was adopted in formalin-fixed, paraffin-embedded sections. Sections were de-paraffinized and incubated with 1% hydrogen peroxide in methanol for 15 min to block the endogenous peroxidase activity. After washed with phosphate-buffered saline (PBS), the sections were incubated with 10% normal rabbit serum for 30 min at room temperature and then incubated with primary antibodies at 1.25 µg/ml for 654-1, 3.6 µg/ml for PG-M1 and 10 µg/ml for control mouse IgG overnight at 4°C. Followed by 3 washes with PBS, the sections were incubated with a second antibody (Histofine SAB-PO (M) kit, Nichirei, Tokyo, Japan) for 120 min at room temperature. After washed with PBS, the sections were then incubated with peroxidase-conjugated streptavidin (Histofine SAB-PO kit) for 30 min at room temperature, and then developed with 3, 3'-diaminobenzidine (Dojindo, Tokyo, Japan) in 0.05M Tris buffer (pH7.64), containing 0.07% sodium azide and 0.02% hydrogen peroxide for 5 min.

Morphometrical analysis

The dThdPase-positive cells were stained brown in cytoplasm and nuclei, occasionally, brown-stained nuclei alone could also be found. CD68-positive cells were also stained brown but a little darker than dThdPase. The number of dThdPase-positive cells and CD68-positive cells was quantified as follows. In each specimen, we selected five fields where the highest density of dThdPase-positive cells was observed after searching the whole field. In each field, immunoreactive cells were counted using an ocular grid (0.25mm×0.25mm) at magnification×400. Then CD68-positive cells were similarly counted in the corresponding five fields in the serial sections. The average number of positive cells in each section was expressed per 0.0625 mm².

Detection of dThdPase protein levels

dThdPase levels in 6 colorectal cancer cells and 2 macrophage-like cells, THP-1 and U937, were measured by ELISA in the study. At least 1×10⁷ cells of each cell line were prepared for this assay and ELISA was performed by Nippon Roche Research Center. It was reported that the dThdPase levels measured by this assay correlated well with a conventional enzyme assay^[19].

Statistical analysis

The significance of differences between means of dThdPase activities in tumor group and normal tissue group was tested by Student's *t* test, and the correlation between the number of dThdPase-positive cells and CD68-positive cells was evaluated

by Spearman's correlation coefficient test, the significance level was at 0.05.

RESULTS

dThdPase activity in tumor and normal mucosal tissues

The dThdPase activity in cancer tissues was 139.7±42.2 (µg 5-FU·hr⁻¹·ml⁻¹), significantly higher as compared with 61.5±21.4 (µg 5-FU·hr⁻¹·ml⁻¹) in the surrounding normal tissues (Figure 1, *P*<0.01).

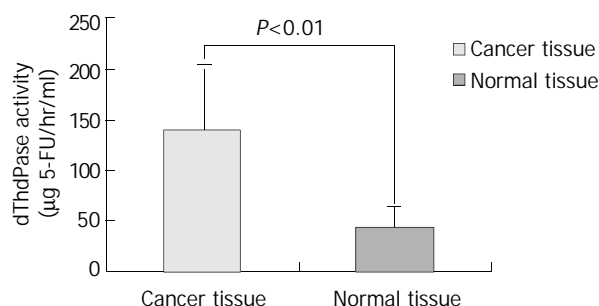


Figure 1 dThdPase activity analysis in 27 specimens of colorectal carcinoma. The dThdPase Activity was 139.7±42.2 (µg 5-FU/hr/ml) in cancer tissues, significantly higher than 61.5±21.4 (µg 5-FU/hr/ml) in adjacent normal tissue (*P*<0.01).

Immunohistochemical staining

In corresponding normal colorectal tissues, epithelial cells at the surface of mucosa were weakly immunoreactive for dThdPase and few stromal cells were positive (Figure 2A). Stromal cells were positive for dThdPase in all cancer tissues, while cancer cells were positive for dThdPase only in 3 out of the 40 cases (Figure 2B). The dThdPase-positive stromal cells were distributed mainly around the cancer nests (Figure 2C) or along the invasive margin of cancer (Figure 2D). In the corresponding areas, CD68-positive macrophages were also increased. The distribution patterns of CD68-positive cells (Figure 2E) were similar to those of the dThdPase-positive stromal cells (Figure 2F).

In the counting analysis of positive cells, the number of dThdPase-positive stromal cells was well correlated with the number of CD68-positive cells (*r*=0.76, *P*<0.0001), (Figure 3).

dThdPase levels in 6 colorectal cancer cell lines and 2 macrophage-like cell lines, were assessed by ELISA. The protein level of dThdPase was 0.5 unit/mg in LS174T, 8.9 unit/mg in Lovo, and not detected in the other 4 colorectal cancer cell lines. In macrophage-like cell lines, 18.2 unit/mg and 19.3 unit/mg of dThdPase were detected in THP-1 and U937, respectively (Table 1).

Table 1 dThdPase levels in 6 colorectal cancers cell lines and macrophage-like cell lines

	dThdPase level* (unit/mg)
Colorectal cancer cells	
LS174T	0.5
Clone A	<0.4 ^a
Colo 320	<1.1 ^a
CX-1	<0.3 ^a
Lovo	8.9
MIP101	<0.2 ^a
Macrophage-like cells	
THP-1	18.2
U937	19.3

*Figures indicate levels of detection limit.

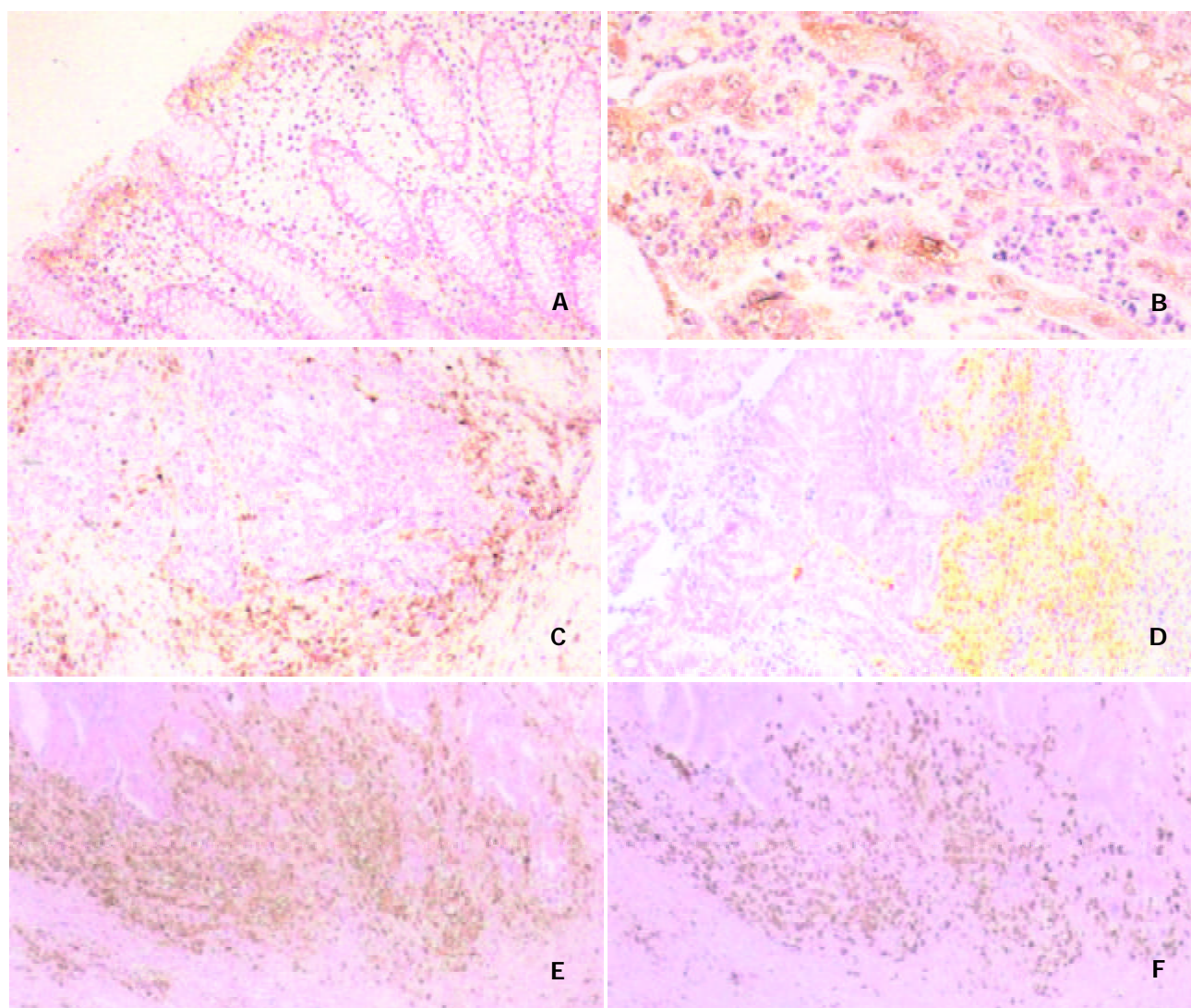


Figure 2 Immunohistochemical staining in colorectal cancer tissues. A: In normal colon mucosa, epithelial cells at the surface are weakly positive for dThdPase and few stromal cells are stained. B: In 3 of the 39 cases examined, cancer cells are positive for dThdPase. C: In most cases of colorectal cancers examined, stromal cells around the cancer nests are positive for dThdPase, while cancer cells are negative for dThdPase. D: In some cases, dThdPase-positive stromal cells are along the invasive margin of cancer, cancer cells are negative for dThdPase. E: Immunohistochemical staining for dThdPase and F: for CD68 in the corresponding area of colorectal cancer tissue. dThdPase-positive stromal cells are observed densely along the invasive margin of cancer. The distribution pattern of dThdPase-positive cells (E) is similar to that of CD68-positive cells (F).

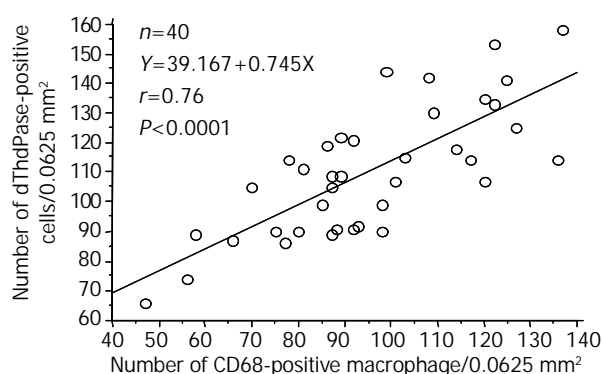


Figure 3 Correlation between number of CD68-positive macrophages and dThdPase-positive stromal cells in 40 cases of colorectal cancer. The significant correlation is judged by Spearman's coefficient rank test ($r=0.76$, $P<0.0001$).

DISCUSSION

It has been well known that dThdPase expression was selectively increased in various malignant tumor tissues

compared with adjacent normal tissues in the same organs^[9-12]. Our data of dThdPase activity analysis in cancer and normal tissues by *ELISA* are also supported the suggestion. However, the present immunohistochemical staining showed that stromal cells around the cancer nests or along the invasive margin of cancer strongly expressed dThdPase activity but few cancer cells did. A significant positive correlation was found between the number of dThdPase-positive stromal cells and CD68-positive macrophages ($r=0.76$, $P<0.0001$). We also detected certain dThdPase levels in macrophage-like cell lines, THP-1 and U937, but seldom detected it in colorectal cancer cell lines by *ELISA*. Takahashi^[20] reported that most cells stained with the same monoclonal antibody (654-1) were also positive for CD68 by double immunohistochemical staining in human colon cancer tissues, while Takebayashi^[9] reported that many colorectal cancer cells were also stained strongly as stromal cells did using the different antibody. These differences in dThdPase stained cancer cells were possibly caused by the different antibodies used, which may recognize the different epitopes. Taken together, TAMs should be a major source for dThdPase expression in colorectal carcinomas, although its mechanism of expression in macrophage is still unclear.

Infiltration of mononuclear inflammatory cells including CD68-positive macrophages has been described as one of the important host reactions in colorectal cancer^[21]. These infiltrating cells are more abundantly distributed along the invasive margin of cancer tissues than in cancer tissues. These reports suggest that CD68-positive TAMs have various roles in interactions between host cells and cancer cells.

Macrophages belong to the mononuclear phagocyte system. They form a heterogeneous cell population and further differentiate into promonocytes and bone marrow monocytes, then enter into blood stream and migrate into tissues, where they undergo final differentiation to tissue macrophages, which are present ubiquitously in all tissues. Besides other functions, such as endocytosis, cytotoxicity, macrophages can secrete over 100 cell products. Many evidences showed that TAMs also appeared to be involved in tumor growth regulation, angiogenesis, host reaction, and antitumor immunity in various malignant tissues by some investigators^[22,23]. TAM has been found to play an important role in tumor angiogenesis by producing a number of angiogenic cytokines, including vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF), transforming growth factor alpha (TGF- α), tumor necrosis factor- α (TNF- α), and PD-ECGF/dThdPase^[24]. Because no macrophage cell line can pass and it is difficult to isolate macrophages, we detected the dThdPase level from macrophage-like cell lines, THP-1 and U937 instead of macrophages,

Using monoclonal antibody anti-CD68 (a macrophage marker), many investigators demonstrated that TAMs were associated with the tumor stage, invasive depth, survival, and angiogenesis in several malignancies^[12,13,15,22,25]. Therefore, CD68 is well known to be a best marker of tissue macrophages. Though it was reported that macrophages could express dThdPase in normal gastrointestinal tract tissues^[26], however, no report so far has dealt with whether colorectal carcinoma cells or macrophages express dThdPase *in vitro*, the mechanism of expression is still unknown.

It is necessary for 5'-DFUR to be converted into 5-FU by dThdPase expressed in cancer tissues during chemotherapy. De Cesare^[27] reported that the effect of 5'-DFUR on human colorectal carcinoma xenografts, it might be due to cytokines induced by TAMs, such as TNF- α , interleukin-1 α (IL-1 α), and interferon- γ (IFN- γ), resulting in an increase of dThdPase activity in cancer tissues. Some investigators have also reported that transfection of dThdPase cDNA could enhance the sensitivity of cancer cells to 5'-DFUR *in vitro*^[28-30], while our another analysis demonstrated that dThdPase was expressed in macrophage-like cells, THP-1, U937, and monocytes could modulate the conversion of 5'-DFUR into 5-FU and exert anticancer effect on 6 colorectal carcinoma cell lines^[31]. Further study is needed to investigate the mechanism, which may provide a future strategy for chemotherapy of colorectal cancer patients with 5'-DFUR.

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