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# Use of Localization and Activity of Thymidine Phosphorylase in Human Gynecological Tumors for Predicting Sensitivity to Pyrimidine Antimetabolite Therapy: An Observational Study

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## Abstract

**Background:** Thymidine phosphorylase (dThdPase) is the rate-limiting enzyme in the conversion of 5′-deoxy-5-fluorouridine (5′-dFUrd), an intermediate metabolite of capecitabine (Xeloda®), to 5-fluorouracil (5-FU). We investigated the correlation between dThdPase activity and immunohistochemical staining in gynecological carcinoma and adjacent normal tissues. We hypothesize that the differential dThdPase activity between tumors and adjacent tissue is predictive of response to treatment with pyrimidine antimetabolites.

**Methods:** In 45 samples of carcinoma tissue and 35 of adjacent normal tissue from 45 patients, we measured dThdPase activity as well as immunoreactivity using an anti-dThdPase monoclonal antibody and macrophage and histiocyte-specific antibodies.

**Results:** dThdPase activity in tumor tissue was significantly higher than that in the corresponding adjacent normal tissue in all samples tested (12 uterine cervical, 19 endometrial, and 4 ovarian tumors). Anti-dThdPase immunopositivity was observed in the epithelial tumor cells of 76.9% of uterine cervical cancer samples, 60.0% of endometrial cancer samples and 63.6% of ovarian cancer samples. In stromal tissue, 84.6% of uterine cervical tumors (11/13), 90.0% of endometrial tumors (18/20), and 81.8% of ovarian tumors (9/11) were immunopositive for anti-dThdPase in interstitial cells (mainly macrophages). Macrophages were also strongly reactive in the stromal tissues of uterine cervical, endometrial, and ovarian cancers. The correlation between dThdPase activity and intensity of immunohistochemical staining of epithelial tumor cells with anti-dThdPase monoclonal antibody was statistically significant in endometrial carcinoma ($P = 0.008$) but borderline in uterine cervical tumors ($P = 0.077$). We found a good correlation between dThdPase activity and staining of epithelial tumor cells, particularly in the case of endometrial cancer.

**Conclusions:** We show that gynecological carcinomas show increased dThdPase activity, and this activity correlates with dThdPase staining of tumor epithelial cells. Thus, dThdPase staining of biopsy specimens could be useful in predicting the outcome of therapy with pyrimidine metabolites.

**Keywords:** Thymidine phosphorylase; Gynecological carcinoma; Pyrimidine antimetabolite therapy

**Abbreviations:** dThdPase: thymidine phosphorylase; 5′-dFUrd: 5′-deoxy-5-fluorouridine; 5-FU: 5-fluorouracil; HPLC: High Performance Liquid Chromatography; mAb: monoclonal antibody; ABC: Avidin-biotin conjugate

## Introduction

Capecitabine (Xeloda®) and 5′-deoxy-5-fluorouridine (5′-dFUrd, Furtulon®) are masked compounds derived from 5-fluorouracil (5-FU) [1]. Thymidine phosphorylase (dThdPase) is the rate-limiting enzyme in the conversion of 5′-deoxy-5-fluorouridine (5′-dFUrd), an intermediate metabolite of capecitabine (Xeloda®), to 5-fluorouracil (5-FU). Although cytostatically inactive by themselves, they exert cytotoxic activity *in vivo* after being converted into 5-FU by the action of pyrimidine nucleoside phosphorylases [2-4], predominantly uridine phosphorylase in mouse and thymidine phosphorylase (dThdPase), reportedly identical to platelet-derived endothelial cell growth factor, in human tumors [5,6]. In addition, 5′-dFUrd is an active intermediate metabolite of capecitabine, which was approved for breast and colorectal cancer in the United States and the European Union. Capecitabine is actively catabolized by dThdPase in humans. From their mechanism of action, the antitumor activity of 5′-dFUrd and

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capecitabine is thought to depend upon the dThdPase activity of cells in tumor tissue, and a positive relationship between their antitumor effect and dThdPase activity has recently been reported [7]. Therefore, presumably, dThdPase values measured in human tumors can be utilized to optimize the efficacy of patients' treatment with 5′-dFUrd and capecitabine. To investigate that possibility, there have been many reports demonstrating dThdPase activity; however, dThdPase activity is dependent on the kind of gynecological cancer.

Analysis of dThdPase levels by high performance liquid chromatography (HPLC) has demonstrated that primary tumor tissues have higher dThdPase activity than adjacent normal tissue of the same organ [2,5,8-13]. Consequently, 5′-dFUrd is preferentially converted to 5-FU in primary tumor tissues after its administration [13]. Among gynecological tumors, however, the relationship between dThdPase activity in the tissues of uterine cervical, uterine endometrial, and ovarian cancers compared with those of adjacent normal tissues has not yet been established. In addition, there have been few reports on the histological localization of dThdPase in gynecological carcinoma tissues. Nevertheless, a report on immunohistochemical staining of uterine cervical carcinoma identified a correlation between survival and immunohistochemical localization of dThdPase [14]. The purpose of the present study is to clarify the relationship between the measured dThdPase activity and its immunohistochemical staining in gynecological cancers.

## Material and Methods

### Patients and samples

Between January 1993 and March 1994, 58 patients from 6 hospitals were enrolled into the present study (15 patients from Kinki University, Osaka; 13 patients from Hyogo Medical Center For Adults, Hyogo; 11 patients from Kurume University, Fukuoka; 9 patients from Jikei University, School of Medicine, Tokyo; 7 patients from Tohoku University, Miyagi; and 3 patients from Tokyo Metropolitan Komagome Hospital, Tokyo, Japan). The investigation was approved by the ethics review committees of all institutions, and all enrolled patients gave their informed consent that their tumor tissue, adjacent normal tissue, and lymph nodes could be used for analysis of dThdPase activity and immunohistochemical and histological examination. All specimens, which consisted of approximately l×l×l-cm samples of primary tumor tissue, adjacent normal tissue, and lymph nodes, were surgically resected and examined by the pathologists at the Department of Pathology, Jikei University School of Medicine. This pathology committee diagnosed histologically malignant tissue involvement in 45 of 58 patients, including 13 patients with uterine cervical carcinoma, 20 with endometrial carcinoma, and 12 with ovarian carcinoma. Histopathological diagnosis was carried out for both the tumor and adjacent normal tissues. Tissue from these 45 patients with malignancy were used for further analysis of dThdPase activity and immunohistochemical assay in the present study.

### Reagents

5-FU was purchased from Kyowa Hakko Kogyo (Tokyo, Japan), and 5′-dFUrd was synthesized at F. Hoffmann-La Roche (Basel, Switzerland). Anti-dThdPase monoclonal antibody (mAb) 654-1 was provided by Nippon Roche Research Center (Kamakura, Japan). Anti-macrophage-CD68 mAb Kp-1 and anti-macrophage-CD68 mAb PG-M1 were purchased from DAKO Co. Ltd. (Glostrup, Denmark) and Vectastain Elite ABC Kit was purchased from Vector Laboratories, Inc. (Burlingame, CA, USA). Diaminobenzidine was purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan).

### Assay of thymidine phosphorylase activity

Tissues were homogenized in 10 mM Tris-HCl buffer (pH 7.4) containing 15 mM NaCl, 1.5 mM MgCl, and 50 mM potassium phosphate. This solution was centrifuged at 105,000 × *g* for 90 min. The supernatant was dialyzed overnight against 20 mM potassium phosphate buffer (pH 7.4) containing 1 mM mercaptoethanol, and then used as a source of crude dThdPase. The protein concentration was determined by the method of Lowry et al. [15]. All procedures were carried out at 4°C. The reaction mixture (120 µ1) for the enzyme activity assay contained 183 mM potassium phosphate buffer (pH 7.4), 10 mM 5′-dFUrd, and the crude enzyme from human tissue. The reaction was carried out at 37°C for 60 min and then terminated by adding 360 µl methanol. The precipitate was removed by centrifugation, and 100 µl supernatant was mixed with 20 µM 5-chlorouracil as the internal standard and applied to a HPLC column (ERC-ODS-1171). The 5-FU was eluted with 50 mM sodium phosphate buffer (pH 6.8) containing 5 mM 1-decanesulfonic acid:methanol (85:15, v/v) and measured with a UV monitor at 280 nm.

### Immunohistochemistry

Tissues taken from the primary tumor lesion, adjacent normal tissue, and lymph nodes were used. Tumors included cancers of the uterine cervix, uterine endometrium, and ovary. Tissues were fixed with 10% formalin in saline for 24 to 72 h, dehydrated, and embedded in paraffin. Sections were cut at 4-µm thickness, placed on slides, deparaffinized in xylene, rehydrated, stained immunohistochemically by using an avidin-biotin conjugate (ABC) system with diaminobenzidine/hydrogen peroxide as substrate, and counterstained with hematoxylin [16]. Slides were cleared and coverslipped for microscopic examination at 200× magnification. The sections were also incubated with primary mAbs [17] at dilutions of 1:4000 for anti-dThdPase, 1:200 for anti-macrophage-CD68 clone Kp-1, and 1:1000 for anti-macrophage-CD68 clone PG-M1, which gave optimal intensity of specific staining with minimal nonspecific background reactivity [18]. The secondary or linking antibody was a biotinylated horse anti-mouse immunoglobulin (Vector Laboratories, Inc., Burlingame, CA). Immunohistochemical intensity was classified into four grades: (-), negative staining; (±), less than 10% positive cells; (+), from more than 10% to less than 50% positive cells; and (++), more than 50% positive cells. Both + and ++ staining were defined as positive, and both ± and - staining were defined as negative.

### Statistical analysis

One and two-sample Wilcoxon tests were performed to detect differences in dThdPase activity between tumor and adjacent normal tissues. Logistic regressions were performed to detect correlations to dThdPase activity by factoring in the number of stained cells per field in addition to the intensity of immunohistochemical staining with anti-dThdPase mAb. All calculations were performed using Windows/SAS 6.12.

## Results

### dThdPase activity in gynecological tumor and normal tissues

Tumor samples obtained from the 45 patients enrolled in this study were analyzed for dThdPase activity, immunohistochemical staining, and histology for diagnosis of the tumor type. Additionally, 35 samples

of normal tissue adjacent to the tumor were also analyzed for dThdPase activity. As shown in Table 1, dThdPase activity in tumor tissue was significantly higher than that in adjacent normal tissue in the 12 cases of uterine cervical cancer ($P = 0.001$, $P = 0.0002$, Wilcoxon one- and two-sample tests, respectively), 19 cases of endometrial carcinoma ($P = 0.0001$, $P = 0.0001$), and 4 cases of ovarian cancer ($P = 0.125$, $P = 0.025$)

| | | dThdPase activity[a)] | | Immunohistochemistry[b)] | | Histopathological |
|---|---|---|---|---|---|---|
| | Case | Tumor | Normal | Epithelial | Stromal | diagnosis[c)] |
| Organ | No. | tissue | tissue | cells | cells | |
| | | | | | | |
| Uterine cervix | 1 | 7.0 | 7.5 | - | + | EC Ad Ca, HD |
| | 2 | 172.5 | NOS[d)] | - | ++ | EC Ad Ca, PUD |
| | 3 | 180.0 | 6.7 | ++ | + | Sq Ca |
| | 4 | 236.0 | 11.0 | ± | ++ | Sq Ca |
| | 5 | 241.0 | 14.2 | ++ | ++ | Sq Ca |
| | 6 | 243.0 | 7.1 | ++ | - | Sq Ca |
| | 7 | 260.6 | 168.2 | ++ | + | Sq Ca |
| | 8 | 341.5 | 1.8 | ++ | + | Sq Ca |
| | 9 | 343.0 | 31.0 | + | + | Sq Ca |
| | 10 | 344.5 | 9.4 | + | + | Sq Ca |
| | 11 | 410.5 | 17.7 | + | ± | EC Ad Ca, PUD |
| | 12 | 572.3 | 26.6 | ++ | ++ | Sq Ca |
| | 13 | 602.5 | 13.4 | ++ | ++ | Sq Ca |
| Endometrium | 1 | 15.1 | 43.9 | - | + | EM Ad Ca |
| | 2 | 20.6 | 28.0 | - | - | EM Ad Ca |
| | 3 | 41.5 | 6.2 | - | + | EM Ad Ca |
| | 4 | 50.8 | 8.4 | + | ++ | EM Ad Ca |
| | 5 | 56.8 | 7.5 | ± | ± | EM Ad Ca |
| | 6 | 70.0 | 1.3 | - | + | EM Ad Ca |
| | 7 | 81.6 | 11.1 | - | + | EM Ad Ca |
| | 8 | 81.8 | 8.3 | - | ++ | Serous Ca |
| | 9 | 111.2 | 73.2 | + | ++ | EM Ad Ca |
| | 10 | 117.6 | 9.2 | + | ++ | EM Ad Ca |
| | 11 | 120.0 | NOS | + | + | EM Ad Ca |
| | 12 | 129.0 | 6.5 | + | ++ | EM Ad Ca |
| | 13 | 135.0 | 5.1 | - | ++ | EM Ad Ca |
| | 14 | 161.4 | 8.3 | + | ++ | EM Ad Ca |
| | 15 | 187.0 | 24.4 | + | ++ | EM Ad Ca |
| | 16 | 192.5 | 59.5 | + | + | EM Ad Ca |
| | 17 | 200.0 | 10.6 | ++ | ++ | EM Ad Ca |
| | 18 | 203.0 | 18.7 | + | + | EM Ad Ca |
| | 19 | 229.0 | 28.6 | + | ++ | Clear cell Ca |
| | 20 | 339.0 | 12.4 | + | ++ | Serous Ca |
| Ovary | 1 | 11.5 | NOS | - | + | Mucinous Ca |
| | 2 | 12.3 | NOS | - | + | Endomet Ca |
| | 3 | 21.3 | NOS | + | + | Clear cell Ca |
| | 4 | 42.5 | 11.6 | + | - | Mucinous Ca |
| | 5 | 48.0 | NOS | - | ++ | Serous cyst Ca |
| | 6 | 61.7 | 0.0 | + | ++ | Mixed epithel Ca |
| | 7 | 73.0 | NOS | + | + | Clear cell Ca |
| | 8 | 75.0 | NOS | ++ | + | Clear cell Ca |
| | 9 | 101.0 | 14.8 | + | - | Clear cell Ca |
| | 10 | 143.0 | NOS | NOS | NOS | Serous cyst Ca |
| | 11 | 238.4 | NOS | - | ++ | Serous cyst Ca |
| | 12 | 12852.5 | 19.3 | ++ | ++ | Serous cyst Ca |

a) dThdPase activity is expressed as μg FU produced per mg protein per hour.
b) Immunohistochemistry is scored by the number of cells showing positive staining for anti-dThdPase mAb:
(-), negative staining; (±), weak and less than 10% positive cells; (+), 10% to 50% positive cells; (++), more than 50% positive cells.
c) EC Ad Ca, endocervical adenocarcinoma; HD, highly differentiated; EMOD, endometrioid carcinoma; PUD,
poorly differentiated adenocarcinoma; Sq Ca, squamous cell carcinoma; EM Ad Ca, endometrial adenocarcinoma; Serous Ca, serous adenocarcinoma; Serous cyst Ca, serous cyst adenocarcinoma; Endomet Ca, endometrial adenocarcinoma; Clear cell Ca, clear cell carcinoma; Mucinous Ca, mucinous cyst adenocarcinoma; Mixed epithel Ca; mixed epithelial adenocarcinoma.
d) NOS, no specimen

**Table 1:** Activity of a pyrimidine nucleoside phosphorylase, dThdPase, immunohistochemistry in gynecological tumors and adjacent normal tissue.

for which normal tissue was available.

### Immunohistochemistry with anti-dThdPase, anti-macrophage-CD68 clone Kp-1, and anti-macrophage-CD68 clone PG-M1 mAbs

We observed positive dThdPase immunostaining of epithelial cells in 76.9% of cervical tumors of the uterus (10/13 samples), 60.0% of endometrial tumors (12/20 samples), and 63.6% of ovarian tumors (7/11 samples), for a mean rate of 66.8% of gynecological cancers demonstrating dThdPase immunopositivity in epithelial cells. Interestingly, in stromal tissue, 84.6% of uterine cervical tumors (11/13), 90.0% of endometrial tumors (18/20), and 81.8% of ovarian tumors (9/11) were immunopositive for anti-dThdPase in interstitial cells (mainly macrophages). In immunohistochemical staining of normal epithelium and stromal tissue, weak reactivity to dThdPase was observed only in the nuclei of basal cells and the cytoplasm of superficial cells. In contrast, in the epithelium of uterine cervical carcinoma, immunoreactivity was present in both the primary carcinoma cells themselves and in the interstitial cells. In squamous cell carcinoma samples, both nuclei and cytoplasm were immunoreactive. The nucleus was stained in superficial carcinoma cells, while cytoplasmic staining was observed in both invasive and parabasal-layer cells (Figure 1). Cytoplasmic staining was often seen in the well-differentiated squamous cell carcinoma. In endocervical adenocarcinoma of the uterus, the highly differentiated type showed cytoplasmic staining of interstitial cells only (data not shown). In endometrial carcinoma, epithelial cells stained weakly with anti-dThdPase mAb (Figure 2). However, the staining was more intense in stromal cells than in epithelial tumor cells. Antimacrophage staining with CD68 and Kp-1 mAbs showed that anti-dThdPase staining in stromal cells was localized mainly to macrophages. In ovarian carcinoma, both serous and mucinous adenocarcinomas showed negative staining of epithelial tumor cells with anti-dThdPase mAb; however, the staining was positive for epithelial cells of clear cell adenocarcinoma (Figure 1). Some of the interstitial cells surrounding carcinoma cells showed stronger staining than the carcinoma cells themselves. Morphologically, the interstitial cells stained with anti-dThdPase mAb appeared to be macrophages or histiocytes in all tumors (Figure 1). They were also immunopositive for anti-macrophage mAb PG-M1 and/or anti-CD68 mAb Kp-1 in endometrial carcinoma, as shown in Figure 2. In the lymph nodes, premature lymphocytes, macrophages, and histiocytes showed strong positive staining, but the lymphocytes themselves were not immunopositive (data not shown).

### Correlation between dThdPase activity and intensity of immunohistochemical staining

Table 2 shows the results of logistic regression analysis of dThdPase activity and the intensity of immunohistochemical staining with anti-dThdPase mAb. A significant correlation was only observed in endometrial carconoma, but not in uterine cervical and ovarian carcinoma.

## Discussion

Increased levels of dThdPase have been reported in many malignant tumors [5,9,12]. Although there have been a several reports that investigated the dThdPase activity in cervical, endometrial and ovarian carcinoma [19-21], it remains to be determined. In this study of gynecological malignant tumors, we found that uterine cervical, endometrial, and ovarian carcinoma also demonstrated higher dThdPase activity than adjacent normal tissue. Several studies of dThdPase expression in cancer have been performed, because this enzyme is thought to activate pyrimidine antimetabolites [6,22]; however, only

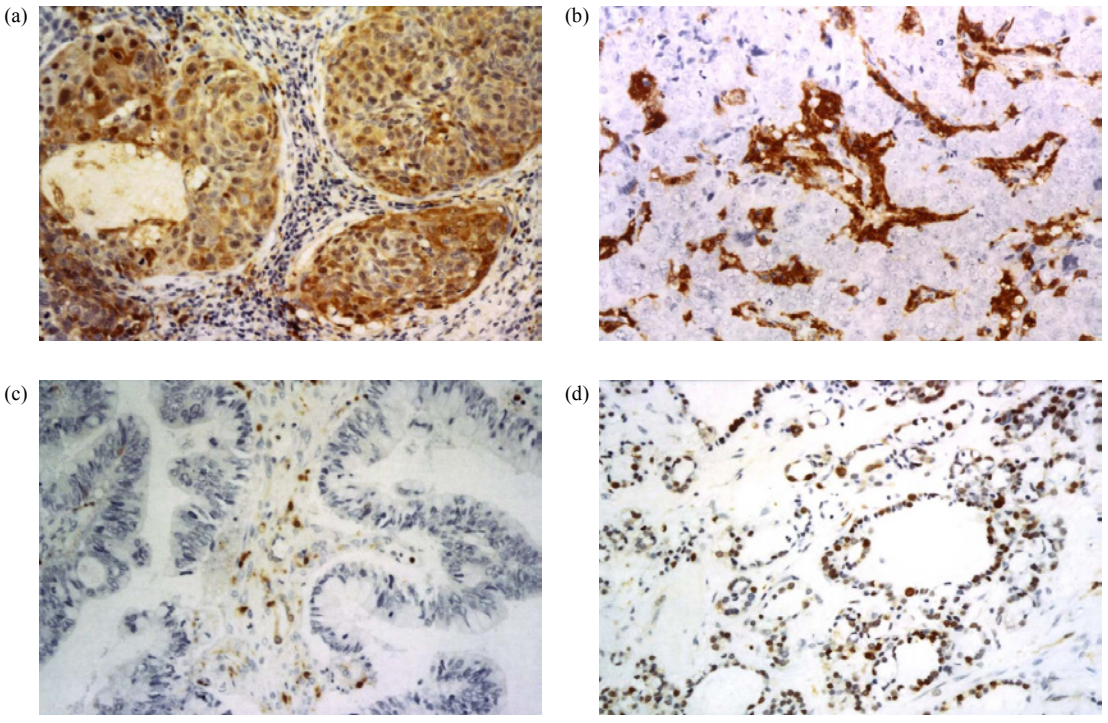


**Figure 1:** Immunohistochemical staining of squamous cell carcinoma with an anti-dThdPase mAb. (a) Uterine cervical carcinoma, (b) a serous papillary carcinoma of the ovary, (c) a mucinous adenocarcinoma of the ovary, and (d) a clear cell adenocarcinoma of the ovary.

a few reports have demonstrated the localization of dThdPase in tumor tissues [14,23,24]. In the present study, immunohistochemical staining with anti-dThdPase mAb revealed that dThdPase was strongly localized to epithelial tumor cells in squamous cell carcinoma of the uterine cervix. In endometrial carcinoma, dThdPase was found to be localized to epithelial tumor cells and, in stromal cells, mainly to macrophages. Serous and mucinous adenocarcinomas, both histologically serous types of ovarian cancer, showed no staining of carcinoma cells themselves. In contrast, in clear cell adenocarcinoma of the ovary, which constitutes less than 20% of ovarian cancer cases, dThdPase was localized to epithelial cells. Therefore, the relatively low dThdPase activity of ovarian cancer may be related to the absence of immunoreactivity in carcinoma cells.

Recently, some investigators have suggested that stromal dThdPase status may be a prognostic factor for survival [14,25,26], and some basic and clinical reports on dThdPase indicate that it is a predictive factor for 5′-dFUrd and capecitabine in the treatment of several carcinomas [25,27]. The results of logistic regression analyses of dThdPase activity and immunohistochemical staining intensity with anti-dThdPase mAb in the present study illustrate that dThdPase activity was well correlated with the intensity of staining in the epithelium in endometrial carcinoma and borderline in cervivcal carcinoma, but not with staining of stromal cells in cervical and ovarian carcinoma. Our resolution power was not sufficient to detect a correlation between dThdPase activity and stromal staining intensity. These results suggest that dThdPase activity reflects the intensity of immunohistochemical staining of epithelial tumor cells, as shown by their correlation, in endometrial carcinoma. The total immunohistochemical intensities did not significantly correlate with their enzymatic activities because of the lack of correlation between stromal cells and immunochemical staining in cervical carcinoma, and between both stromal and epithelial cells and immunochemical staining in ovarian carcinoma. Only a few reports that showed the good correlation between dThdPase activity and immunochemical staining in gynecologic cancer have been published [28,29], and it should be confirmed in other studies. In case of cervical carcinoma, since almost all squamous cell carcinomas showed very high dThdPase activity, the statistical correlation may have been obscured. The statistical correlation between dThdPase activity and intensity if immunohistochemistry was proven only for endometrial carcinomas, and that of uterine cervix was borderline. For ovarian carcinoma, data are sparse. dThdPase gene expression was proven to be significantly high in ovarian carcinoma [29] as shown in our study. Although good dThdPase activity and immunochemical staining were well correlated besed on the past reports [19], our study did not show the correlation. The number of cases may have been too small for sufficient statistical power.

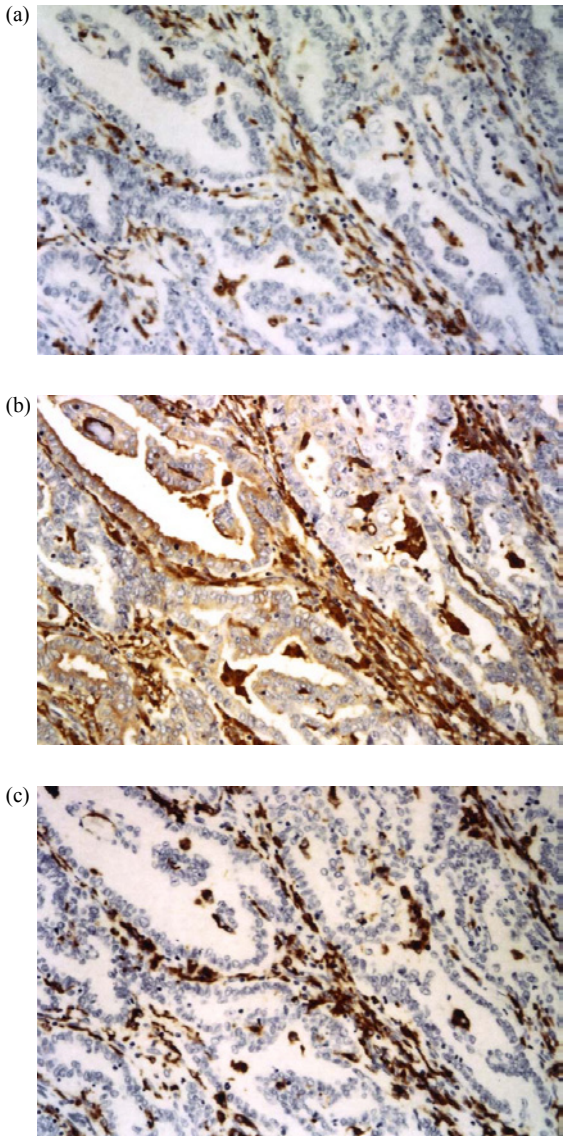


**Figure 2:** Immunohistochemical staining of an endometrial adenocarcinoma by the indirect method with ABC. (a) With anti-macrophage (PG-M1) mAb, (b) with anti-dThdPase mAb, and (c) with anti-CD68(Kp-1)mAb.

| | | Epithelial cells | | | Stromal cells | |
|---|---|---|---|---|---|---|
| | *P*-value | Odds ratio | (95% CI) | *P*-value | Odds ratio | (95% CI) |
| Uterine cervix | 0.077 | 1.009 | (0.999-1.019) | 0.432 | 1.003 | (0.996-1.010) |
| Endometrium | 0.008 | 1.023 | (1.006-1.041) | 0.039 | 1.017 | (1.001-1.034) |
| Ovary | 0.185 | 1.005 | (0.997-1.014) | 0.308 | 1.011 | (0.990-1.033) |

**Table 2:** Results of logistic regression of dThdPase activity and intensity of immunohistochemical staining with anti-dThdPase mAb.

## Conclusion

We investigated the correlation between dThdPase activity and immunohistochemical staining in gynecological carcinoma and adjacent normal tissues. Our hypothesis is that the differential dThdPase activity between tumors and adjacent tissue will be predictive of response to treatment with pyrimidine antimetabolites. We show that gynecological carcinomas show increased dThdPase activity, and this activity correlates with dThdPase staining of tumor epithelial cells. Thus, dThdPase staining of biopsy specimens might be useful in predicting the outcome of therapy with pyrimidine metabolites.

### Competing Interests

The authors declare that they have no competing interests.

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