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# The Expression Level of Human Thymidine Phosphorylase in Urinary Tract Cancer

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**Human thymidine phosphorylase (dThdPase) catalyses reversible phosphorylation of thymidine to deoxyribose-1-phosphate and thymine, and is identical to Platelet-derived endothelial cell growth factor (PD-ECGF), which is an angiogenic factor purified from human platelet. In this study, we determined dThdPase expression levels in urinary tract cancer by enzyme-linked immunosorbent assay and determined whether they correlated with tumor stage and grade in bladder cancer. The mean level of dThdPase expression in cancer tissue was higher than in normal tissue in bladder cancer (41.1±50.7 unit/mg protein vs 17.6±17.8 unit/mg protein) and in upper urinary tract cancer (52.4±53.1 unit/mg protein vs 17.6±17.8 unit/mg protein). dThdPase expression level was correlated with tumor grade and stage in bladder cancer. These data suggest that dThdPase/PD-ECGF is an important angiogenic factor for growth and extension of urinary tract cancer.**

**Key words :** human thymidine phosphorylase (dThdPase); platelet derived endothelial cell growth factor (PD-ECGF); transitional cell carcinoma

## Introduction

Tumor angiogenesis is the formation of new vessels toward and within a tumor, resulting in tumor growth and metastasis. Recently, there have appeared several reports concerning angiogenesis in bladder cancer. Tumor angiogenesis was reported as an independent prognostic indicator for patients with invasive transitional cell carcinoma of the bladder in a microvessel density study[1,2]. Increased expression of basic fibroblast growth factor and vascular endothelial cell growth factor, which are well-known angiogenic growth factors have been recognized in bladder cancer[3,4].

Human thymidine phosphorylase (dThdPase), an enzyme involved in pyrymidine nucleotide metabolism, is known to be identical with Platelet-derived endothelial cell growth factor (PD-ECGF), an angiogenic factor[5]. PD-ECGF is expressed in macrophages, stromal cells, and glial cells of normal tissue and is not expressed in normal gastrointestinal epithelium, bladder epithelium, or smooth muscle by the immunohistochemical method[6]. The PD-ECGF/dThdPase expression levels in several kinds of cancer (colon, breast, and gastric) were higher than those in the surrounding normal tissues[7,8]. In this study, we investigated PD-ECGF/dThdPase expression levels and sites of dThdPase/PD-ECGF in urinary tract cancer and surrounding normal tissues using enzyme-linked immunosorbent assay (ELISA) and immunohistochemical methods.

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## Patients and methods

### *Patients*

Specimens were obtained from 13 patients (11 men, 2 women) with upper urinary tract transitional cell carcinoma (TCC) and 35 patients (27 men, 8 women) with bladder TCC. The pathological stage and grade of the tumor were diagnosed by special pathologists according to TNM criteria.

### *Reagent*

Mouse monoclonal antibody MoAb 104B, MoAb 232-2 and MoAb 654-1, which recognizes human dThdPase, was kindly provided by Nippon Roche Co. Ltd., Tokyo, Japan. These monoclonal antibodies were prepared using dThdPase purified from human colon cancer xenograft HCT 116 in mice[9].

### *ELISA*

Tumor and normal epithelial tissues were obtained from each patient and were packed in ice, and stored at -80℃ until use for ELISA. Each tissue was homogenized in 10 mM Tris-HCl buffer (pH 7.4) containing 15 mM NaCl, 1.5 mM $MgCl_2$ and 50 mM potassium phosphate, and then

centrifuged at 105000 xg for 90 min. The supernatant was dialyzed overnight against 20 mM potassium phosphate buffer (pH 7.4) and 1 mM 2-mercaptethanol, and was then used as a souse of crude dThdPase[9]. The protein concentration was determined by the method descrived by Lowry et al[10]. The amount of dThdPase was calibrated with that measured for standard solutions, and was evaluated as unit/tissue protein volume (mg)[9].

A 96-well microtiter plate (Nunc-immuno-plate Maxisorp, Nunc, Roskilde, Denmark) was incubated at 4℃ overnight with 10 $\mu$g/ml of the dThdPase MoAb 104B in 10 mM phosphate buffered saline solution (PBS, pH 7.6). The plate was coated with 3% (w/v) skim milk in PBS (blocking buffer) for 1 hour at room temperature. The plate was washed with PBS containing 0.05% Tween 20 and 0.05% sodium azide and kept at 4℃ until use. Test samples and standard solutions of dThdPase, which are HCT 116 tumor homogenates serially diluted with a blocking buffer, were dispensed onto the plate coated with antibody. The plate was incubated [1] at 37℃ for 1 hour and then washed with 0.05% Tween 20 in PBS ; [2] incubated with MoAb 232-2 at 1 $\mu$g/ml in blocking buffer for 1 hour at 37℃ and washed ; [3] incubated with 2000-fold diluted anti-mouse IgG conjugated with horseradish peroxidase (Bio-Rad, Hercules, CA) for 30 min at 37℃ and washed ; [4] incubated with a substrate solution containing 3,3',5,5'-tetramethylbenzidine (TMB) and $H_2O_2$ (TMB microwell peroxidase substrate system, KPL, Goithersgurs, MD) for 10 to 20 min at room temperature. The peroxidase reaction was stopped by the addition of 1 M phosphate solution, and the amount of dThdPase sandwiched with the two anti-dThdPase MoAb was estimated by measuring its absorbency at 450 nm with a plate reader (Bio-Rad, model 3550).

*PD-ECGF/dThdPase Immunohistochemistory*

Formalin-fixed paraffin-embedded sections were placed on silan coated glass slides (MASTUNAMI, Japan). The deparaffinized sections were placed in 0.1 M citrate buffer (pH 6.0) and heated twice in a microwave oven for 5 min. The primary antibody MoAb 654-1 was applied at a dilution of 1 : 1000 and visualized by the alkaline phosphotase anti-alkaline phosphotase method. The slides were counterstained with 2% methylgreen and mounted.

*Statistics*

The relationships between dThdPase expression levels and categorical variables were evaluated using the Mann-Whitney U test and t test. A P value of less than 0.05 was considered statistically significant.

## Results

*PD-ECGF/dThdPase expression in transitional cell carcinoma and surrounding normal tissue*

PD-ECGF/dThdPase expression of bladder cancer tissue was higher than that of the normal tissue (41.1 ± 50.7 unit/mg protein vs 17.6 ± 17.8 unit/mg protein). The expression level in upper urinary tract cancer tissue was also higher than that of the normal tissue (52.4 ± 53.1 unit/mg protein vs 17.6 ± 17.8 unit/mg protein) (Fig. 1). There was a significant difference between cancer and normal tissue.

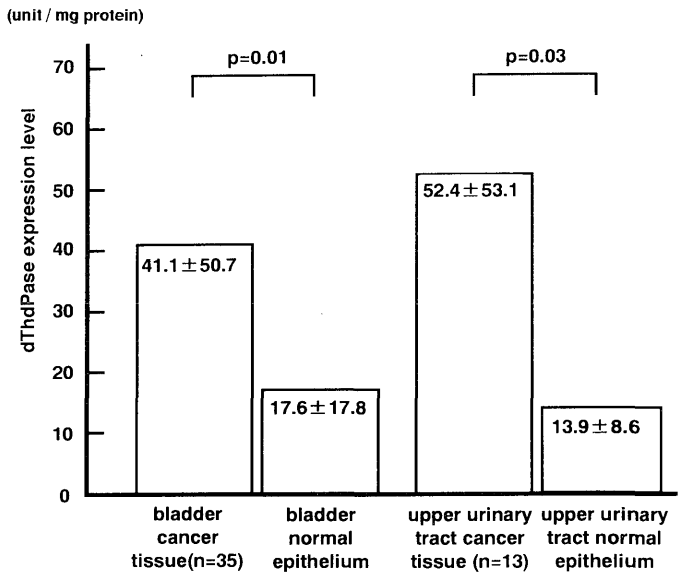


**Fig. 1** dThdPase expression level in normal epithelial tissue and cancer tissue.

*Relationship between dThdPase expression level and pathological stage and grade in bladder cancer*

The mean expression levels were 94.8 ± 80.5 unit/mg protein in invasive tumor, 30.5 ± 22.6 unit/mg protein in T1 tumor, and 9.9 ± 7.7 unit/mg protein in Ta tumor (Fig. 2). Thus, the level of dThdPase expression was increased in progressing stages of bladder cancer. There was a significant difference in dThdPase expression level between Ta and T1 ($p=0.04$), as well as between Ta and invasive tumor ($p=0.02$). The level of dThdPase expression in G3 bladder cancer (61.9 ± 64.9 unit/mg protein) was 3 times as high as in G1 (20.4 ± 12.9 unit/mg protein) and twice as high as in G2 (34.7 ± 47.0 unit/mg protein). There was a significant difference in dThdPase expression between G1 and G2 or G3. Elevation of dThdPase expression was found to parallel increases in histologic grade and progression of bladder cancer (Fig. 3).

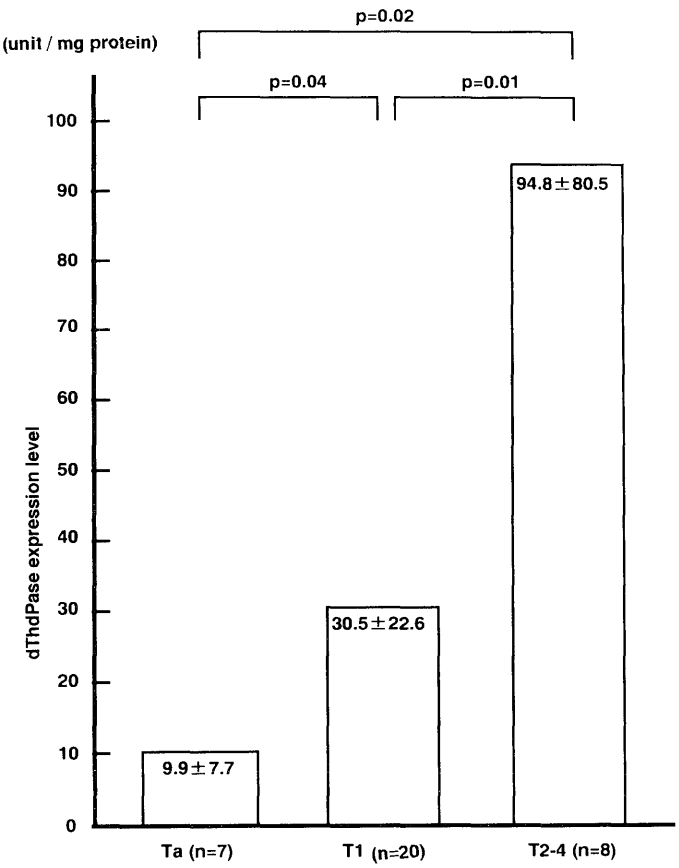


**Fig. 2** dThdPase expression level in superficial (stage Ta and T1) and invasive (stage T2-T4) bladder cancer.

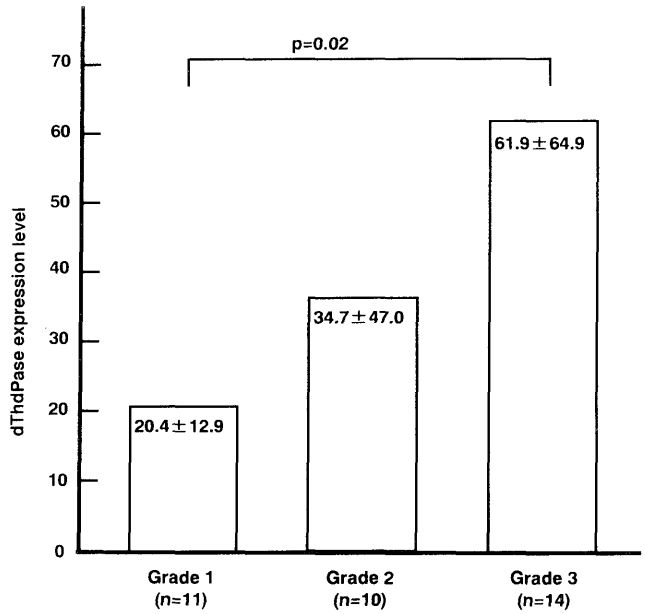


**Fig. 3** dThdPase expression level in bladder cancer of different grades.

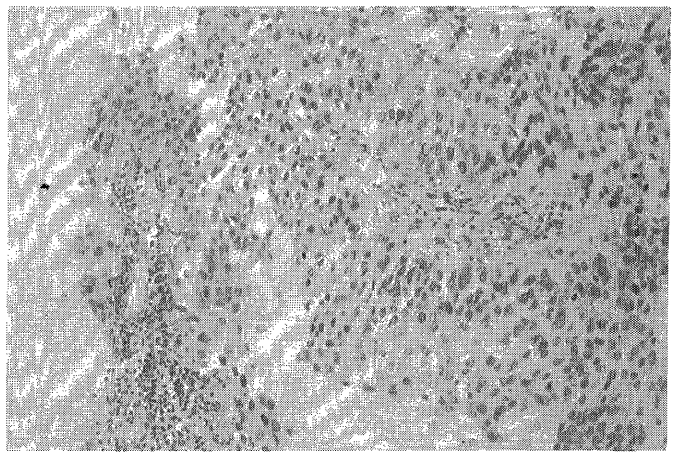

**Fig. 4-a** Section from the bladder cancer specimen, showing both nucleus and cytoplasm expressing for PD-ECGF/dThdPase (x200).

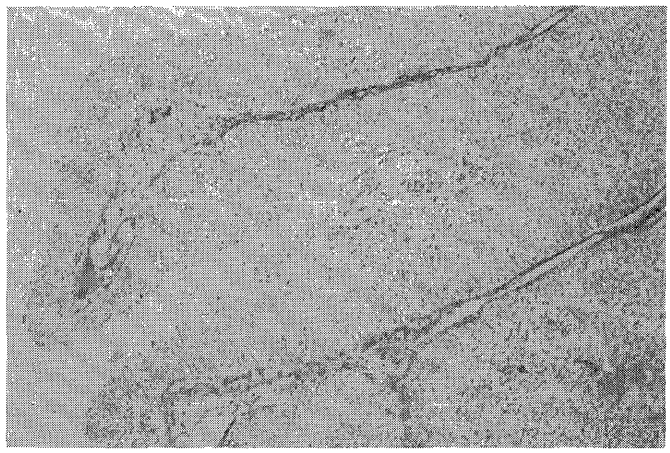

**Fig. 4-b** Interstitial cells in a superficial bladder cancer specimen were positively stained for PD-ECGF/dThdPase, and cancer cells were not stained (x100).

*Immunohistochemistory*

The usual pattern of PD-ECGF/dThdPase expression was primarily seen in cytoplasm and sometimes in both the nucleus and cytoplasm in cancer cells (Fig. 4-a). Normal epithelial cells were not stained for PD-ECGF/dThdPase. The interstitial cells and the endothelial cells in tumor vessels were positively stained for PD-ECGF/dThdPase in several patients (Fig. 4-b). Furthermore, dThdPase immunoreactivity in interstitial cells was observed in patients with highly dThdPase expression.

## Discussion

There are 2 previous studies about the relationship between PD-ECGF/dTdRPase expression and tumor grade and stage in bladder cancer. Kubota et al. examined

dThdPase activity by enzyme assay, and Mizutani et al. examined levels of PD-ECGF expression by high performance liquid chromatography and enzyme-linked immunosorbent assay[11,12]. As in our study, their findings suggested a correlation between PD-ECGF/dTdRPase expression and tumor stage and grade. In the present study, the author demonstrated that the expression of dThdPase was increased in urinary tract cancer compared with surrounding normal tissue. In bladder cancer, stage progression and increased pathological grade correlated with dThdPase expression. Furthermore, the expression of dThdPase in T1 bladder cancer was significantly higher than that in Ta bladder cancer, and there were no significant differences between Ta cancer tissue and normal epithelium. These results suggested that submucosal infiltration of cancer cells may be the first step in inducing the expression of dThdPase. However, further studies are needed to clarify the relatoinship between dThdPase expression and tumor extension.

Angiogenesis is induced by various angiogenic factors produced by cancer cells or non-malignant cells that infiltrate the cancer. In this study, dThdPase immunoreactivity was observed not only in cancer cells but also in interstitial cells in patients with highly dThdPase expressing. These results suggest that dThdPase/PD-ECGF may be produced by cancer cells and interstitial cells in urinary tract cancer.

5'-Deoxy-5-fuluorouridine (5'-dFUrd : Furtulon®) exhibits antitumor activity through its conversion to 5-fluorouracil by dThdPase[9]. Clinically, high stages and grades of urinary tract cancer have more malignant potential, and a high incidence of recurrence and progression is a serious problem. In this study, a high-stage and high-grade tumor expressed a high level of dThdPase. Therefore, treatment with 5'-dFUrd may be effective in these tumors.

In conclusion, the current study demonstrated that the expression of dThdPase/PD-ECGF was increased in urinary tract cancer compared with normal tissue, and elevation of dThdPase/PD-ECGF expression correlated with progression stage and grade increase in bladder cancer.

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