

## Expression of Thymidine Phosphorylase in Primary Human Renal Cell Carcinoma by ELISA Method

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**Thymidine phosphorylase (TP) expression in 100 paired samples of renal cell carcinoma (RCC) and normal adjacent tissue was analyzed by an ELISA method. We also investigated whether TP expression correlates with clinicopathological findings and clinical outcomes of these patients. Median TP expression was 9-fold (range, 0.5–56) higher in primary tumor than in non-cancerous renal tissue ( $P < 0.0001$ ). There was a significant difference with respect to tumor venous invasion. TP expression was significantly higher in patients with such venous invasion than in those without ( $P = 0.018$ ). However, there was no correlation between TP level and other clinicopathological findings and the survival curves. These results suggest that ELISA is useful for evaluating TP expression of human RCC and may provide a novel approach to therapy for patients with RCC.**

Key words: Renal cell carcinoma — Thymidine phosphorylase — ELISA

Angiogenesis, the process of new blood vessel formation, is necessary for normal growth and development.<sup>1)</sup> Active angiogenesis rarely occurs in the normal adult, except during monthly uterine changes, placentation, and in wounds. Malignant solid tumors also require neovascularity for growth, progression, and metastasis.<sup>2)</sup> In cancers, increased vascular density reportedly correlates with a higher incidence of metastasis and a poor prognosis.<sup>3–6)</sup>

Tumor angiogenesis involves interactions between endothelial cells, tumor cells, tumor-infiltrating leukocytes, and the extracellular matrix.<sup>7)</sup> Angiogenesis is regulated by many angiogenic factors, such as vascular endothelial growth factor,<sup>8,9)</sup> angioprotein-1,<sup>10)</sup> platelet-derived endothelial cell growth factor (PD-ECGF),<sup>11)</sup> basic-fibroblast growth factor,<sup>12)</sup> hepatocyte growth factor,<sup>13)</sup> IL-8,<sup>14)</sup> proliferin,<sup>15)</sup> and angiogenin.<sup>16)</sup>

PD-ECGF was originally identified as the factor in platelet lysates which stimulates proliferation of aortic endothelial cells *in vitro*.<sup>11)</sup> Thymidine phosphorylase (TP) is involved in pyrimidine nucleoside metabolism. TP catalyzes the reversible phosphorolysis of thymidine, deoxyuridine, and their analogs to their respective bases and 2-deoxyribose-1-phosphate.<sup>17,18)</sup> Recently, PD-ECGF was identified as TP.<sup>19)</sup> The exact mechanism of angiogenesis by TP is still unknown, but TP shows angiogenic activity *in vivo* and its enzymatic activity is indispensable for its angiogenic activity. Transfection of TP into transformed fibroblasts in nude mice increases tumor vascularization.<sup>20)</sup> TP hydrolysis of thymidine gives rise to 2-deoxy-D-ribose-

1-phosphate, which is readily dephosphorylated to 2-deoxy-D-ribose. The latter has an angiogenic function.<sup>21)</sup> Although the lysates of COS-7 cells transfected with the TP cDNA have angiogenic activity, those transfected with mutant TP cDNA did not. Moreover, angiogenic activity of TP is inhibited by the 6-amino-5-chlorouracil moiety of a TP inhibitor.<sup>22)</sup>

Nishida *et al.* developed an ELISA method using a monoclonal antibody to measure the amount of TP, which showed a good correlation with the enzymatic activity.<sup>23)</sup> In our study, we analyzed TP expression in 100 tumor/non-cancerous tissue pairs of primary renal cell carcinoma (RCC) using ELISA. The relationships between TP expression, clinicopathological findings, and prognostic value were evaluated.

### MATERIALS AND METHODS

**Human tissue samples** Human tissue samples from 100 Japanese patients with RCC treated at Chiba University Hospital were obtained from surgery between November 1991 and October 1997. No patient had received preoperative radiation therapy or chemotherapy. Patients' average age at surgery was 59 years (range, 35–92). Paired samples from primary tumors and non-cancerous kidney tissue were obtained from all cases. After nephrectomy, all specimens were immediately stored at  $-80^{\circ}\text{C}$ . Both cancerous and normal adjacent tissues were removed and confirmed histologically. Each tumor was classified according to the tumor-node-metastasis (TNM) grading system<sup>24)</sup> and the criteria of the Japanese Urological Association.<sup>25)</sup> Patients' characteristics are shown in Table I. Mean follow-up in

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Table I. Patients' Characteristics and TP Expression in Renal Cell Carcinoma

Factor	Cases	Average±SD (U/mg protein)	P value
Sex			NS
male	70	104.2±79.1	
female	30	87.2±60.9	
Tumor status			NS
pT1a	33	103.1±86.0	
pT1b	29	90.7±80.5	
pT2	3	125.0±14.2	
pT3	33	101.5±62.3	
pT4	2	100.9±45.8	
Tumor grade			NS
grade 1	44	87.1±68.9	
grade 2	41	114.9±81.8	
grade 3	15	90.9±63.5	
Histologic type			NS
renal cell carcinoma			
clear cell	79		
granular	3		
chromophobe	4		
spindle cell	5		
collecting-duct carcinoma	9	71.4±70.2	
Tumor size			NS
<4 cm	35	104.5±83.6	
4 cm ≤ , <7 cm	39	97.3±79.6	
7 cm ≤	26	94.5±51.1	
Infiltration pattern			NS
INFα	49	101.4±81.6	
INFβ	38	110.9±92.3	
INFγ	13	92.7±67.8	
Cell growth pattern			NS
Expansive type	66	110.1±90.0	
Infiltrating type	34	100.1±85.2	
Node status			NS
pN0	94	99.9±74.5	
pN1, pN2	6	86.5±75.6	
Venous invasion			P=0.018
negative	54	85.7±69.0	
positive	46	114.8±77.0	
Distant metastasis			NS
negative	97	98.9±74.8	
positive	3	103.7±64.2	
TNM stage			NS
I	62	97.5±83.3	
II	3	125.0±14.2	
III	29	98.7±59.0	
IV	6	115.2±72.0	

NS, not significant.

these cases was 52 months (1–111 months). Eight of the 100 patients died of RCC and 2 died of other diseases. The other 90 patients lived throughout the follow-up period.

**ELISA** ELISA was done according to the reported method.<sup>23</sup> Tissue samples were homogenized with 10 volumes of 10 mM Tris-HCl buffer (pH 7.4) containing 15 mM NaCl, 1.5 mM MgCl<sub>2</sub> and 0.05 mM phosphate-buffered saline (PBS, pH 7.6) in a glass homogenizer. The homogenates were centrifuged at 10 000g for 15 min. The supernatants were stored at -80°C until use. A 96-well microtiter plate (Nunc Immunoplate Maxisorp, Nunc, Roskilde, Denmark) was incubated at 4°C overnight with 10 μg/ml of the TP monoclonal antibody (MoAb) 104B in 10 mM PBS. The plate coated with the antibody was then incubated with 3% (w/v) skim milk in PBS for 1 h at room temperature. The plate was washed with PBS containing 0.05% Tween 20 and 0.05% sodium azide and kept at 4°C until used. Test samples and standard solutions of serially diluted recombinant TP, the concentration of which was calibrated in ELISA with a known amount of TP activity in the homogenate of HCT116 tumor xenografts. The plate was incubated at 37°C for 2 h and then washed with 0.05% Tween 20 in PBS; incubated with TP MoAb 232-2 at 1 μg/ml in blocking buffer for 2 h at 37°C and washed; incubated with 2000-fold diluted anti-mouse IgG conjugated with horseradish peroxidase (Bio-Rad, Hercules, CA) for 1 h at 37°C and washed; and incubated with a substrate solution containing 3,3',5,5'-tetramethylbenzidine (TMB) and H<sub>2</sub>O<sub>2</sub> (TMB microwell peroxidase substrate system, KPL) for 5 to 10 min at room temperature. The peroxidase reaction was stopped by the addition of 1 M phosphate solution, and the amount of TP sandwiched with the two anti-TP MoAbs was estimated by measuring its absorbance at 450 nm with a plate reader (Bio-Rad, model 3550). The amount of TP was determined from a calibration curve prepared with standard solutions. There was a good correlation between TP found by ELISA and the enzyme activity.

**Statistical analysis** The levels of TP expression in tumors and in non-cancerous renal tissues were compared using the Wilcoxon single rank test. The Mann-Whitney *U* test or Kruskal-Wallis test was used for evaluation of the relationship between the level of TP expression and the clinicopathological features. Survival curves were calculated using the Kaplan-Meier method and analyzed by using the log-rank test and the generalized Wilcoxon test. Statistical significance was defined as *P*<0.05.

## RESULTS

The TP expression levels were evaluated by ELISA in normal tissues adjacent to the cancer and cancer tissues of surgically resected specimens which were obtained from

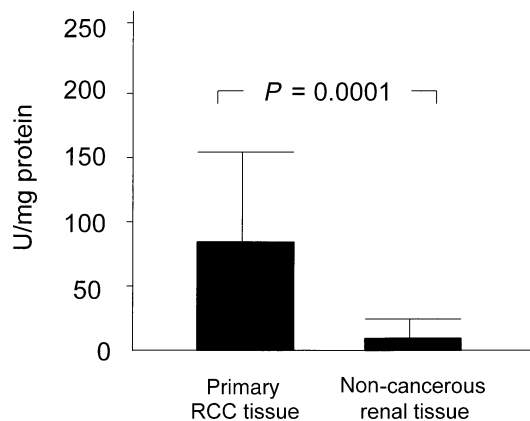


Fig. 1. TP expression in normal tissues adjacent to tumor and in cancerous tissue analyzed by ELISA. One unit corresponds to the TP level of the standard enzyme solution (extract of human colon cancer xenograft HCT 116), which converts 5'-dFUrd to 5-FUra at the rate of 1  $\mu$ g 5-FUra/h. RCC, renal cell carcinoma.

100 patients with RCC. The TP expression levels were significantly higher in cancer tissues than in matched non-cancerous renal tissues. Median TP expression was 9-fold (range, 0.5–56) higher in the primary tumor (median, 76.3 U/mg protein; range, 2.7–381.8 U/mg protein) than in non-cancerous renal tissue (median, 8.2 U/mg protein; range, 1.5–91.3 U/mg protein;  $P < 0.0001$ ; Fig. 1).

Furthermore, we analyzed the association between the TP expression levels in RCC and clinicopathological parameters including tumor status, tumor grade, tumor size, histological type, lymph node metastasis, venous invasion, distant metastasis, and TNM stage in Table I. A

significant difference was noted with respect to tumor venous invasion. The TP expression was significantly higher in patients with such venous invasion than in those without ( $P = 0.018$ ). These results suggest that this method is useful not only for predicting the clinical efficiency of 5'-deoxy-5-fluorouridine (5'-dFUrd), but also for understanding the roles of this enzyme in angiogenesis in RCC tissues. However, there was no significant association between the TP expression level and sex or the clinicopathological characteristics including tumor status, tumor grade, histological type, lymph node metastasis, distant metastasis, and TNM stage.

To evaluate progression-free survival and overall survival based on the TP expression of RCC, the 100 patients with RCC were divided into two groups at the median value (high TP, 76.3 U/mg protein  $\leq$  TP value; low TP, TP value  $<$  76.3 U/mg protein). As shown in Fig. 2A, there was no relationship between the TP expression level and progression-free survival. Next, we also evaluated overall survival in these two groups. The 10-year overall survival rate of the patients with low TP cancers was 82.1%, whereas that of patients with high TP cancers was only 46.4%. Although there is no significant relation between the TP expression level and overall survival, these results suggest that high TP expression of RCC is closely related to the malignant character of the affected tumor, which strongly influences the postoperative course of the patients.

**DISCUSSION**

Using ELISA, we demonstrated greater TP levels in primary RCC than in paired non-cancerous renal tissue.

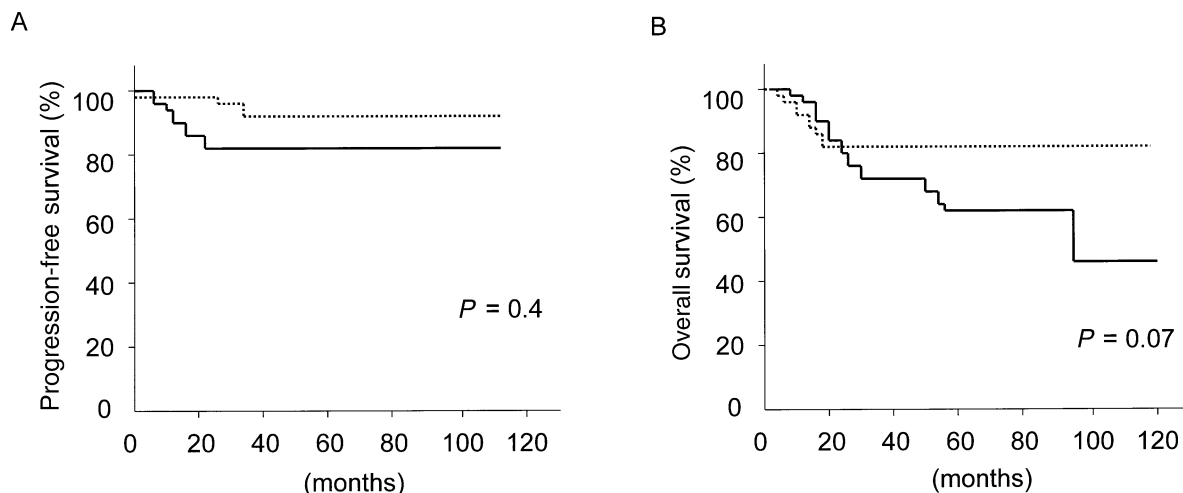


Fig. 2. Progression-free survival (A) and overall survival (B) in the low TP-expressing and high TP-expressing groups among all patients with renal cell carcinoma. ---- low TP, — high TP.

Many investigators have reported that TP activity or TP expression in various solid tumors is higher than in the normal adjacent tissues, such as urinary bladder, ovary, stomach, and colon.<sup>26–29)</sup> O'Brien *et al.* reported that mRNA expression of TP in invasive bladder cancers was 260-fold and 33-fold higher in superficial tumor than in normal bladder tissues, respectively.<sup>26)</sup> In another report, the median TP expression level in bladder carcinoma was 2.6-fold higher than in normal bladder tissue.<sup>27)</sup> Concerning RCC, Imazono *et al.* reported that the median TP enzymatic activity in RCC was 9-fold higher than in non-cancerous renal tissue, and there was a significant correlation between TP expression and microvessel density.<sup>30)</sup> The present study revealed that TP expression in primary RCC was elevated 9-fold compared to that of paired adjacent normal tissue. These data suggest that TP expression plays an important role in the neovascularization of some human RCC.

The TP expression correlates significantly with various clinicopathological factors. TP expression levels in the tumor tissues, such as esophagus, stomach, colon, and lung, were reported to be elevated as the cancer stage progressed.<sup>29)</sup> Imazono *et al.* reported that tumor cell TP immunoreactivity correlates with RCC tumor grade, but there is no correlation between tumor cell TP expression and other clinicopathological features.<sup>30)</sup> In our study, the TP expression found by ELISA was significantly higher in patients with venous invasion than in those without. It is unknown whether TP directly participates in venous invasion, but this result suggests that it does have some influence.

Since angiogenesis is considered to be a potent prognostic factor,<sup>3,6,31)</sup> the value of TP expression in relation to prognosis is of interest. Higher levels of TP expression are associated with poor clinical prognosis in colorectal carcinoma.<sup>32)</sup> Patients with TP-positive gastric carcinoma have a significantly worse prognosis than those with TP-negative carcinoma, overall and in stage III.<sup>33,34)</sup> TP overexpression was found to be an independent prognostic factor in multivariate analysis of RCC.<sup>30)</sup> Patients with TP-positive

tumors have a significantly worse prognosis than those with TP-negative tumors.<sup>34)</sup> It has also been reported that multivariate analysis of RCC showed a significant correlation between survival and TP expression, but angiogenesis did not correlate with TP expression or survival.<sup>35)</sup> On the other hand, O'Brien *et al.* found no correlation between tumor cell TP expression and relapse-free survival or overall survival in bladder carcinoma.<sup>36)</sup> Fox *et al.* also reported that TP expression does not correlate with prognosis in all patients.<sup>37)</sup> In our study, we could not obtain conclusive results on the prognostic value of TP expression in renal carcinomas, since our follow-up periods are rather short to evaluate progression-free or overall survival. TP expression may not be a prognostic marker, but may perhaps be a predictive marker for response to chemotherapy. The up-regulation of TP expression in primary RCC identifies it as a therapeutic target. 5'-dFUrd ("Furtulon") is a prodrug of 5'-fluorouracil (5'-FUra) and TP converts it to 5'-FUra.<sup>38,39)</sup> The advantages of the ELISA method include the small sample weight required (10 mg) and the ability to analyze a large number of samples. The results of this method correlate well with conventional enzyme assays, such as HPLC.<sup>23)</sup> Thus, tumor TP measurement might indicate likely response to treatment, and highly selective targeting of 5-fluorouracil (5-FU) to tumor tissue would thereby be achieved. Yamamoto *et al.* reported that advanced breast cancer patients with a TP-positive primary tumor responded well to 5'-dFUrd.<sup>40)</sup> Moreover, evaluation of TP expression might be helpful in selecting adjuvant chemotherapy for gastric cancer.<sup>41)</sup>

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