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The Significance of Thymidine Phosphorylase/ Platelet-Derived Endothelial Cell Growth Factor Activity in Renal Cell Carcinoma

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BACKGROUND. Thymidine phosphorylase (TP) is identical to platelet-derived endothelial cell growth factor (PDECGF) and has angiogenic activity. Little is known about the significance of TP/PDECGF activity in patients with renal cell carcinoma (RCC). The authors examined the enzymatic activity of TP in 66 RCC specimens and investigated the association between the level of TP activity and the stage/grade status of patients with RCC. Furthermore, the authors examined the correlation between TP/PDECGF activity and prognosis.

METHODS. TP activity levels in nonfixed, fresh-frozen RCC specimens and in specimens of normal kidney were determined using a thin-layer chromatography assay.

RESULTS. The activity of TP was approximately 3.5-fold greater in RCC specimens compared with normal kidney specimens. TP activity in patients with Stage III–IV RCC was 2.6-fold greater compared with TP activity in patients with Stage I–II RCC. In addition, the level of TP activity was correlated with a higher grade of RCC. Patients who had RCC with low TP activity had a longer postoperative disease-specific survival compared with patients who had RCC with high TP activity in the 5-year follow-up.

CONCLUSIONS. The current study is the first to demonstrate a correlation between levels of TP activity and both disease progression and a higher grade of RCC. It also is the first to show that elevated TP activity in patients with RCC predicts a poor prognosis. The results suggest that high TP/PDECGF activity may be associated with the malignant potential of RCC and that TP/PDECGF may be a molecular therapeutic target in patients with RCC. ***Cancer* 2003;98:730–6.**

KEYWORDS: renal cell carcinoma, thymidine phosphorylase, platelet-derived endothelial cell growth factor, vascular endothelial growth factor, 5-fluorouracil.

Angiogenesis is prerequisite to tumor growth and metastasis and is induced by various angiogenic factors produced by tumor cells and/or nonmalignant cells that infiltrate the tumor.[1,2] It has been shown that increased vascular density is correlated with a greater incidence of metastasis and a worse prognosis in various types of malignant disease, including renal cell carcinoma (RCC).[3,4] Although numerous angiogenic factors have been described, the relative importance of individual angiogenic factors in most tumor types remains largely unclear.[5,6] In patients with RCC, several reports have described increased expression of basic fibroblast growth factor, placental growth factor, and vascular endothelial growth factor (VEGF).[7–9]

Platelet-derived endothelial cell growth factor (PDECGF) is a potent angiogenic factor and has been identified as thymidine phosphorylase (TP).[10,11] The mechanism responsible for its angiogenic action remains unclear but is dependent on its enzymatic action.[12] Hydrolysis of thymidine by TP gives rise to 2-deoxyribose 6-phosphate that is dephosphorylated readily to 2-deoxyribose, which reportedly is angiogenic.[13] The expression of TP/PDECGF is elevated in several solid tumors.[14,15] The increased expression of TP/PDECGF was found to be correlated with the degree of malignancy in ovarian carcinoma, and transfection of TP/PDECGF into breast carcinoma cells promoted tumor cell growth.[12,15] Although TP/PDECGF is an important enzyme for angiogenesis, the reported data on TP/PDECGF activity in RCC are limited, and little is known about the significance of TP/PDECGF activity in the biology of RCC.[16] In the current study, the activity of TP/PDECGF was measured in 66 RCC specimens, and the correlation between the level of TP/PDECGF activity and the stage/grade status of patients with RCC was evaluated. Furthermore, the association between TP/PDECGF activity and prognosis was examined.

MATERIALS AND METHODS

Patients

Surgical specimens were obtained from 66 patients with RCC. They included 49 male patients and 17 female patients who ranged in age from 32 years to 82 years. Histologic diagnoses revealed that 61 patients had clear cell RCC, and 5 patients had papillary RCC. Their histologic classification and staging according to the TNM classification system[17] were as follows: 46 patients had T1 tumors, 10 patients had T2 tumors, 9 patients had T3 tumors, and 1 patient had a T4 tumor; 62 patients had N0 lymph node status, 2 patients had N1 lymph node status, and 2 patients had N2 lymph node status; 60 patients had M0 disease, and 6 patients had M1 disease. There were 46 patients with Stage I RCC, 8 patients with Stage II RCC, 5 patients with Stage III RCC, and 7 patients with Stage IV RCC. Eleven patients had Grade 1 tumors, 49 patients had Grade 2 tumors, and 6 patients had Grade 3 tumors. Samples of normal kidney also were collected from the same 66 patients with RCC. The specimens were stored frozen at −80 °C until they were used for the assay of TP activity. The current study was performed after approval by the local Human Investigations Committee. Informed consent was obtained from each patient.

Measurement of TP Activity in RCC and Normal Kidney

TP activity was measured by determining the conversion of labeled thymidine to labeled nucleotides using the thin-layer chromatography (TLC) method, as described previously.[18,19] RCC and normal kidney samples were homogenized in 4 volumes of 50 mM Tris-HCl, pH 8.0, containing 5 mM 2-mercaptoethanol, 25 mM KCl, and 5 mM $MgCl_2$ and were centrifuged at 105,000 × *g* at 4 °C for 60 minutes in an ultracentrifuge (Model TL-100; Beckman Instruments, Fullerton, CA). The supernatants from each sample were divided into several tubes and frozen at −80 °C until use.

The reaction mixture, in a total volume of 100 μL, consisted of 100 mM sodium phosphate buffer, pH 6.4; 2.5 mM β-mercaptoethanol; 0.5 mM [^{3}H]thymidine; and the supernatant (50 μL). The mixture was incubated for 20 minutes at 37 °C, and the reaction was stopped by heating at 100 °C in a water bath. After centrifugation at 14,000 rpm for 2 minutes, the supernatant (20 μL) was applied to a polyethyleneimine cellulose TLC sheet and was developed with distilled water. The spots corresponding to the substrate and product were visualized under ultraviolet light and excised, and the level of radioactivity in each was measured. Internal standards were used to compare assays. This method made it possible to estimate TP activity > 0.01 nmol/mg protein per minute. We analyzed all samples at the same time. TP activity greater than the median value was regarded as high activity, and TP activity less than the median value was regarded as low activity.

Enzyme-Linked Immunosorbent Assay for VEGF Expression

The expression of VEGF protein was quantitated by sandwich enzyme-linked immunosorbent assay.[20,21] Briefly, 100 μL of each sample and its 3 dilutions (3:4, 1:2, and 1:4) or serial dilutions of standard VEGF protein (Nippon Roche Company Ltd., Tokyo, Japan and Otsuka Assay Company Ltd., Tokushima, Japan) was added to 96-well, flat-bottom microtiter plates coated with anti-VEGF monoclonal antibody (mAb) (Nippon Roche Company Ltd. and Otsuka Assay Company Ltd.). After a 1-hour incubation at room temperature, the plates were washed 3 times with phosphate-buffered saline (PBS), and 100 μL of peroxidase-conjugated anti-VEGF mAb solution (Nippon Roche Company Ltd. and Otsuka Assay Company Ltd.) was added and incubated for another hour at room temperature. After washing 3 times with PBS, 100 μL of reaction mixture (1.0 mM tetramethylbenzidine peroxidase substrate, 10 mM citric acid, and 3.5 mM H_2O) was added for the color reaction. After incubation for 1

hour at room temperature, 2 N H_2SO_4 was added to each well to terminate the enzyme reaction and to stabilize the developed color. The optical density was measured at 450 nm using a microculture plate reader (Immunoreader; Japan Intermed Company Ltd., Tokyo, Japan). The concentrations of VEGF protein were calibrated from a dose-response curve based on reference standards. We measured the concentration of standard VEGF protein five times for the standard curve. Internal standards were used to compare assays. This method made it possible to estimate VEGF expression $>$ 5 pg/mg protein. Repeated measurements yielded the same results.

Reagents and Medium

5-fluorouracil (5-FU: lot. no. 308033) was kindly supplied by Kyowa Hakkou Company Ltd. (Tokyo, Japan). RPMI-1640 medium (Bio-cult; Gibco, Glasgow, Scotland, United Kingdom) supplemented with 25 mM N-2-hydroxyethyl-piper-az-ine-N′-2-ethane-sulphonate (HEPES; Gibco), 2 mM L-glutamine (Gibco), 1% nonessential amino acid (Gibco), 100 U/mL penicillin (Gibco), 100 μg/mL streptomycin (Gibco), and 10% heat-inactivated fetal bovine serum (Gibco) was used as complete medium.

Tumor Cells

Fresh RCC cells derived from 23 patients with RCC were separated from surgical specimens for in vitro primary culture, as described previously.[22,23] Briefly, cell suspensions were prepared by treating finely minced tumor tissues with collagenase (3 mg/mL; Sigma Chemical Company, St. Louis, MO). After washing in RPMI-1640 medium, the cell suspensions were layered on discontinuous gradients consisting of 2 mL of 100% Ficoll-Hypaque, 2 mL of 80% Ficoll-Hypaque, and 2 mL of 50% Ficoll-Hypaque in 15-mL plastic tubes and were centrifuged at $\times$ 400 *g* for 30 minutes. Lymphocyte-rich mononuclear cells were collected from the 100% interface, and tumor cells and mesothelial cells were collected from the 80% interface. Cell suspensions enriched with tumor cells sometimes were contaminated by monocyte-macrophages, mesothelial cells, or lymphocytes. To eliminate further contamination of host cells, we layered the cell suspensions on discontinuous gradients of 2 mL each of 25% Percoll, 15% Percoll, and 10% Percoll in complete medium in 15-mL plastic tubes and centrifuged them for 7 minutes at 25 $\times$ *g* and room temperature. Tumor cells depleted of lymphoid cells were collected from the bottom, washed, and suspended in complete medium. Tumor cells were $>$ 93% viable on average according to the trypan blue dye-exclusion test. The tumor cells were maintained in monolayers on plastic dishes in complete medium. The tumor cells from primary culture were used as target cells for lysis of 5-FU in the microculture tetrazolium dye (MTT) assay.

Cytotoxicity Assay

The MTT assay was used to determine tumor cell lysis by 5-FU, as described previously.[24,25] Briefly, 100 μL of target cell suspension (2×10^4 cells) was added to each well of 96-well, flat-bottom microtiter plates (Corning Glass Works, Corning, NY), and each plate was incubated for 24 hours at 37 °C in a humidified 5% CO_2 atmosphere. After incubation, the supernatant was aspirated, tumor cells were washed 3 times with RPMI medium, and 200 μL of drug solution or complete medium for control was applied to the 96-well plates. Each plate was incubated for 24 hours at 37 °C. After incubation, 20 μL of MTT working solution (5 mg/mL; Sigma Chemical Company) was added to each culture well, and the cultures were incubated for 4 hours at 37 °C in a humidified 5% CO_2 atmosphere. The culture medium was removed from the wells and replaced with 100 μL of isopropanol (Sigma Chemical Company) supplemented with 0.05 N HCl. The absorbance of each well was measured with a microculture plate reader (Immunoreader; Japan Intermed Company Ltd.) at 540 nm. The percent cytotoxicity was calculated using the following formula: percent cytotoxicity = [1 $-$ (absorbance of experimental wells/absorbance of control wells)] $\times$ 100.

Statistical Analysis

All determinations were made in triplicate. For statistical analysis, Student *t* tests, Pearson correlation tests, one-way analyses of variance, and multiple comparison tests were used. Postoperative disease-specific survival was determined by the Kaplan–Meier method. The Cox–Mantel test and generalized Wilcoxon tests were used to establish the statistical difference in survival between patients with high levels and low levels of TP activity. Factors related to disease-specific survival in patients with RCC also were analyzed by multivariate analysis. A P value $\leq$ 0.05 was considered significant.

RESULTS

TP Activity Levels in RCC and Normal Kidney

The levels of TP activity in RCC and normal kidney in patients with RCC are summarized in Figure 1. The mean TP activity in RCC was approximately 3.5-fold greater compared with the activity in normal kidney (confidence interval, 2.3–4.7). The median levels of TP activity in normal kidney and RCC were 0.33 nmol/mg protein per minute (range, 0.02–1.64 nmol/mg protein per minute) and 1.07 nmol/mg protein per minute

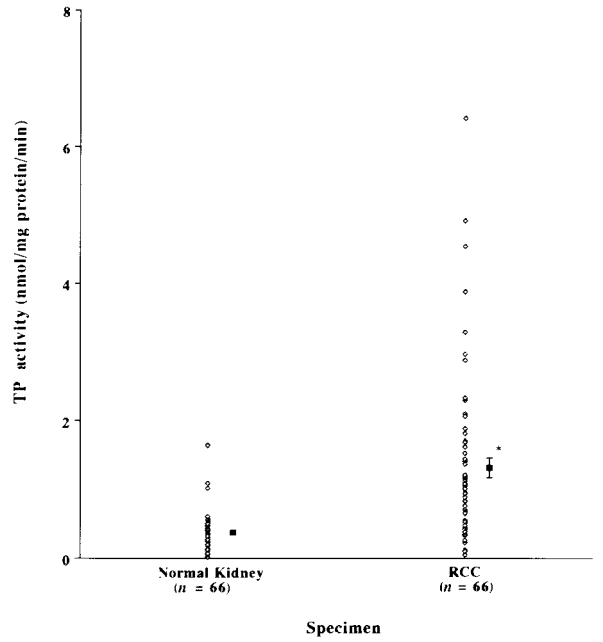

FIGURE 1. Levels of thymidine phosphorylase (TP) activity in renal cell carcinoma (RCC) and normal kidney. TP activity in RCC and normal kidney was quantitated with the thin-layer chromatography assay, as described in the text (see Materials and Methods). Squares indicate mean values. Error bars are ± standard error. An asterisk indicates $P < 0.05$ compared with normal kidney (paired Student t test).

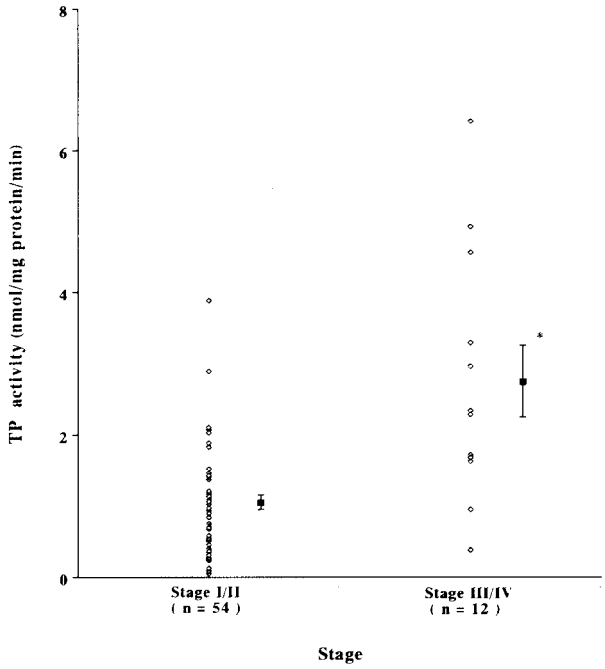

FIGURE 2. Levels of thymidine phosphorylase (TP) activity according to stage grouping in patients with renal cell carcinoma (RCC). The levels of TP activity in RCC were quantitated with the thin-layer chromatography assay, as described in the text (see Materials and Methods). Squares indicate mean values. Error bars are ± standard error. An asterisk indicates $P < 0.05$ compared with patients who had Stage I–II disease (two-tailed Student t test).

(range, 0.06–6.42 nmol/mg protein per minute), respectively. The level of TP activity in normal kidney in patients with RCC was similar to that in patients with renal pelvic carcinoma or ureteral carcinoma (data not shown). These findings demonstrated that TP activity in RCC was significantly greater than TP activity in normal kidney.

TP Activity Levels in RCC

We then examined TP activity in RCC as a function of histologic disease stage and tumor grade. The mean level of TP activity in patients with Stage III–IV RCC was 2.6 times greater than the TP activity in patients with Stage I–II RCC (Fig. 2). The median levels of TP activity in patients with Stage I–II RCC and Stage III–IV RCC were 0.97 nmol/mg protein per minute (range, 0.06–3.89 nmol/mg protein per minute) and 2.34 nmol/mg protein per minute (range, 0.39–6.42 nmol/mg protein per minute), respectively. The mean level of TP activity in patients with T3–T4 RCC was approximately three times greater than the TP activity in patients with T1–T2 RCC. Furthermore, the mean level of TP activity in patients with M1 RCC was significantly greater (by three times) than the TP activity in patients with M0 RCC (data not shown).

The mean level of TP activity in patients with Grade 2 RCC was two times greater than the TP activity in patients with Grade 1 RCC (Fig. 3), and the mean level of TP activity in patients with Grade 3 RCC was two times greater than the TP activity in patients with Grade 2 RCC. The median levels of TP activity in patients with Grade 1, Grade 2, and Grace 3 RCC were 0.96 nmol/mg protein per minute (range, 0.06–1.20 nmol/mg protein per minute), 1.07 nmol/mg protein per minute (range, 0.12–6.42 nmol/mg protein per minute), and 2.98 nmol/mg protein per minute (range, 2.11–3.89 nmol/mg protein per minute), respectively. The levels of TP activity in patients with clear cell RCC were similar to the levels of TP activity in patients with papillary RCC (data not shown).

Correlation between Levels of TP Activity and Postoperative Disease-Specific Survival in Patients with RCC

Patients with RCC who underwent radical nephrectomy were evaluated for their postoperative clinical course. The postoperative disease-specific survival was estimated using Kaplan–Meier analysis. Based on that analysis, patients with RCC were divided into two

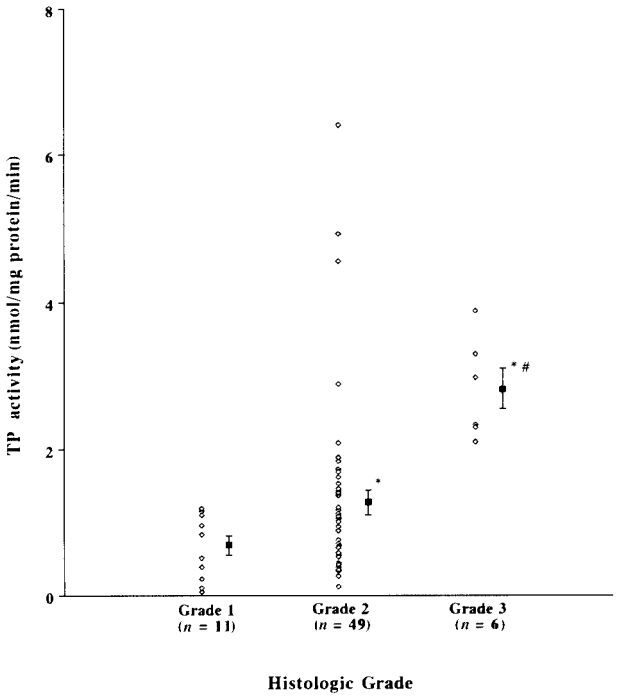

FIGURE 3. Levels of thymidine phosphorylase (TP) activity according to histologic grade of renal cell carcinoma (RCC). The levels of TP activity in RCC were quantitated using the thin-layer chromatography assay, as described in the text (see Materials and Methods). Squares indicate mean values. Error bars are ± standard error. Asterisks indicate $P < 0.05$ (Grade 1 vs. Grade 2 and Grade 1 vs. Grade 3). The pound sign indicates $P < 0.05$ (Grade 2 vs. Grade 3; one-way analysis of variance and multiple-comparison test).

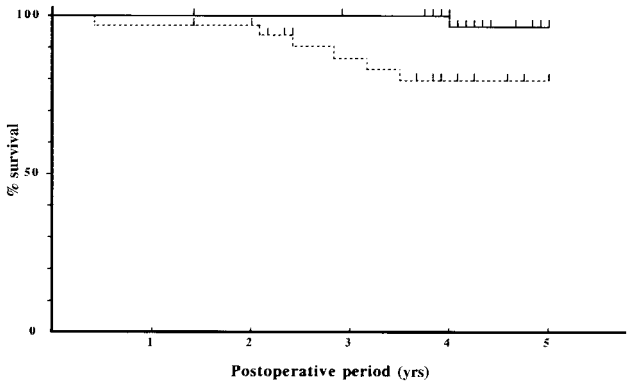

FIGURE 4. Correlation between the level of thymidine phosphorylase (TP) activity and postoperative disease-specific survival in patients with renal cell carcinoma (RCC). The postoperative disease-specific survival of patients with RCC who underwent radical nephrectomy was determined using the Kaplan–Meier method. TP activity greater than the median value was regarded as high activity, and TP activity less than the median value was regarded as low activity. There was a significant difference in disease-specific survival between the following groups in the 5-year follow-up ($P < 0.05$; generalized Wilcoxon test and Cox–Mantel test): 33 patients with low TP activity (solid line) and 33 patients with high TP activity (dashed line).

groups: those with high TP activity (greater than the median value) and those with low TP activity (less than the median value). Patients with low TP activity had a longer disease-specific survival compared with patients who had high TP activity at the 5-year follow-up (Fig. 4). However, the Cox proportional hazards regression model and multivariate analysis showed that TP activity was not an independent prognostic factor in patients with RCC.

DISCUSSION

In the current study, we demonstrated that the activity of TP/PDECGF was up-regulated in RCC compared with normal kidney and that the elevation of TP/PDECGF activity was correlated with stage progression and increases in tumor grade in patients with RCC. Furthermore, to our knowledge, this study is the first to show that patients who had RCC with low TP activity had a longer disease-specific survival compared with patients who had high TP activity at the 5-year follow-up. Although the current study reports on a small number of patients during a short-term follow-up, the findings suggest that TP/PDECGF may play an important role in regulating the malignant potential of RCC and may be of prognostic value in patients with RCC. Low TP activity may be considered a good prognostic sign.

PDECGF originally was identified as the angiogenic factor in platelet lysates that stimulated proliferation of aortic endothelial cells in vitro and was shown to be homologous to TP.[10,11] TP/PDECGF is a dimmer of identical subunits, each of 45 kilodaltons.[2] The molecule lacks a secretion peptide and, in this regard, is similar to acidic and basic fibroblast growth factors. However, TP/PDECGF differs from many endothelial mitogens in that it lacks heparin-binding domains. TP/PDECGF catalyzes the reversible breakdown of thymidine to thymine in the presence of orthophosphate. TP/PDECGF has been identified in several human tissues, with the highest level found in the liver. The level of TP/PDECGF also is elevated in a variety of human malignancies, and increased expression reportedly has been correlated with the degree of malignancy in patients with ovarian carcinoma.[14,15] Transfection of TP/PDECGF into MCF7 breast carcinoma cells promoted tumor growth in vivo.[12] The current study demonstrated that the elevation of TP/PDECGF activity paralleled an increase in histologic disease stage and tumor grade in patients with RCC. Furthermore, the level of TP activity in patients with RCC predicted clinical outcome. These results suggest that TP/PDECGF may be important in the establish-

ment, growth, invasion, and metastasis of RCC. However, further studies are needed to determine the biologic interaction between TP/PDECGF and growth modulation of RCC in vivo.

The current study is the first study to demonstrate that TP enzymatic activity in RCC was significantly greater than TP activity in normal kidney and that the elevated levels of TP activity were correlated with high stage/grade status in patients with RCC. These findings are consistent with other reports demonstrating that TP protein expression in RCC was greater compared with TP protein expression in normal kidney.[16,26] However, those authors did not demonstrate a significant correlation between TP protein expression and tumor stage/grade status in patients with RCC, and their data are not consistent with our findings. Fundamentally, the expression of protein does not always show the activity of the protein, which is the most important indicator in biology. These findings suggest that the measurement of TP enzymatic activity, but not TP protein expression, may be important.

One of the most studied angiogenic factors is VEGF, which is a specific mitogen for endothelial cells and promotes angiogenesis by stimulating capillary proliferation, migration, and permeability. VEGF has been associated positively with a poor prognosis and worsened stage/grade in patients with RCC. Preliminary experiments demonstrated that there was an inverse correlation between TP/PDECGF activity and VEGF expression, although the correlation was modest (data not shown). Those findings suggest that TP/PDECGF and VEGF may compensate each other in angiogenesis in RCC, and these two angiogenic pathways may characterize RCC. Because TP/PDECGF was up-regulated in patients with metastatic or locally advanced, high-grade RCC, TP/PDECGF appears to be associated with a more aggressive phenotype of RCC. It is believed that metastatic RCC arises from cells that are shed from the primary tumor and then implant in metastatic sites. It is reasonable to assume that clones of cells that overexpress TP/PDECGF can establish a blood supply and grow more easily and rapidly after implantation compared with clones that do not overexpress TP/PDECGF. Accordingly, the inhibition of TP/PDECGF may provide a therapeutic means of preventing metastasis of RCC. In addition, the simultaneous inhibition of VEGF may be necessary.

Metastasis and recurrence of RCC remain major problems. Therefore, new therapeutic approaches are required for patients with metastatic and recurrent RCC. The dramatic up-regulation of TP activity in RCC compared with normal kidney, especially in patients with high-stage and high-grade RCC, identifies TP as a molecular therapeutic target. 5-FU is an anticancer prodrug and requires its intracellular conversion to an active metabolite, 5-fluoro-2′-deoxyuridine 5′-monophosphate (FdUMP), by several enzymes, including TP. FdUMP shows its cytotoxicity through the formation of a ternary complex with thymidylate synthase (TS) and 5,10-methylenetetrahydrofolate, resulting in the inhibition of TS and the blocking of DNA synthesis.[27,28] Thus, TP may enhance the cytotoxic activity of 5-FU. In fact, our preliminary observation showed that TP activity in RCC cells was correlated positively with their sensitivity to 5-FU in vitro (data not shown). The correlation between elevated TP activity in RCC and high 5-FU sensitivity may be of potential clinical importance in the management of patients with RCC. Chemoimmunotherapy that includes 5-FU may be effective against RCC in patients with high levels of TP activity. In addition, a prodrug of 5-FU, capecitabine, which is activated by TP, has been developed recently.[29] Capecitabine may be another candidate drug for the treatment of patients with RCC who have high levels of TP activity. The other candidate agent for RCC is a new competitive TP inhibitor, 5-chloro-6-[1-(2-iminopyrrolidinyl)methyl]uracil hydrochloride, which we recently synthesized.[30] However, the mechanisms responsible for 5-FU resistance in tumor cells are multifactorial. Furthermore, 5-FU-containing chemoimmunotherapy is not always effective clinically against RCC. These findings suggest that overcoming 5-FU resistance of RCC or patient selection also may be necessary in the treatment of patients with RCC using 5-FU and its derivatives.

In conclusion, the current study demonstrated that the level of TP/PDECGF activity in RCC was parallel with increases in the histologic disease stage and tumor grade and that elevated levels of TP activity in patients with RCC were associated with a poor prognosis. These findings suggest that the assessment of TP/PDECGF may be useful in both the management and the treatment of patients with RCC. Because the level of TP/PDECGF activity may be used as a prognostic parameter in patients with RCC and as a predictive indicator for 5-FU efficacy against RCC, the accurate prediction of prognosis and 5-FU efficacy may help select patients for more intensive surgical or immunochemotherapeutic approaches, including 5-FU. However, further studies are needed to determine the regulatory effects of TP/PDECGF in patients with RCC.

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