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ORIGINAL PAPER

**Yasuhiro Hirano · Tatsuya Takayama
Shinji Kageyama · Tomomi Ushiyama
Kazuo Suzuki · Kimio Fujita**

Thymidine phosphorylase activity in renal cell carcinoma: relationship between histological parameters and chemosensitivity to fluorouracil-related drugs

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Abstract Thymidine phosphorylase (TdR-Pase) is an essential enzyme in the metabolism of fluorouracil-related drugs and is also a potent angiogenic factor. We measured the TdR-Pase activity in human renal cell carcinomas (RCCs) to assess the relationship between TdR-Pase activity and the degree of tumor malignancy. We also investigated the relationship between TdR-Pase activity and chemosensitivity to fluorouracil-related drugs. A total of 61 RCC tissue specimens and 39 normal kidney tissue specimens were obtained. TdR-Pase activity was measured by enzyme-linked immunosorbent assay. The in vitro histoculture drug response assay was also performed to examine tumor sensitivity to 5-fluorouracil (5-FU) and doxifluridine (5′-DFUR). The TdR-Pase activity of RCCs was 13-fold that of the normal tissues. Activity increased with histological grade, and was significantly higher in high-stage tumors (T3 or higher). TdR-Pase activity exhibited a significant positive correlation with sensitivity to 5-FU and 5′-DFUR. High TdR-Pase activity in human RCC was confirmed to predict high tumor grade and stage. RCCs with high TdR-Pase activity were also sensitive to FU-related drugs.

Keywords Thymidine phosphorylase · Renal cell carcinoma · Chemosensitivity · 5-fluorouracil

Introduction

Thymidine phosphorylase (TdR-Pase) is known to be identical to platelet-derived endothelial cell growth factor (PDECGF), which has a potent angiogenic activity [6]. High levels of this enzymatic activity have been observed in various malignant tumors [4, 5, 15]. A correlation between the expression of this enzyme and the prognosis of human renal cell carcinoma (RCC) has also been observed [5, 14].

TdR-Pase is also one of the key enzymes involved in the metabolism of fluorouracil-related drugs, since it is important in the initial process of 5-FU activation. Doxifluridine (5′-DFUR), a pro-drug of 5-FU, is converted to 5-FU by TdR-Pase. Thus, carcinoma cells with high TdR-Pase activity might be more sensitive to fluorouracil. If the sensitivity to FU-related drugs could be predicted by measuring TdR-Pase activity, this might be a good clinical parameter for choosing drugs for chemotherapy. However, there have only been a few reports published concerning the relationship between the TdR-Pase activity of RCC and sensitivity to FU-related drugs [2, 10].

In this study, we measured the TdR-Pase activity in clinical specimens obtained from human RCCs and confirmed the correlation between TdR-Pase activity and the degree of tumor malignancy. We also investigated the chemosensitivity of tumors to FU-related drugs using an in vitro histoculture drug response assay and compared the results with TdR-Pase activity.

Materials and methods

Cancer specimens

Surgical specimens were obtained from 61 RCC patients at Hamamatsu University Hospital and related hospitals between August 1996 and January 2000. Informed consent to study TdR-Pase activity and chemosensitivity was obtained from all patients before surgery. All of the cancer tissue specimens were histologically confirmed to be RCC. Histological grades and stages were classified according to the 1997 International Union Against Cancer TNM classification and were as follows: grade (G) 1: $n=16$, G 2: $n=40$, and G 3: $n=5$; T1: $n=16$, T2: $n=31$, T3: $n=13$, and T4: $n=1$.

Y. Hirano (✉) · T. Takayama · S. Kageyama
T. Ushiyama · K. Suzuki · K. Fujita
Department of Urology,
Hamamatsu University School of Medicine,
431-3192, 1-20-1 Handayama Hamamatsu City, Japan
E-mail: hirano@hama-med.ac.jp
Tel.: +81-53-4352306
Fax: +81-53-4352305

Normal kidney tissue

Samples of normal kidney tissue were obtained from 39 nephrectomy specimens. An area far from the RCC was selected and approximately 1 cm^3 of tissue was removed. A small portion of each sample was then screened by H and E staining to ensure that the specimens contained no carcinoma.

Enzyme-linked immunosorbent assay for TdR-Pase activity

Specimens were rapidly frozen and stored at –80°C until assayed. The tissue was homogenized in 10 mM Tris-HCl buffer (pH 7.4) containing 15 mM NaCl, 1.5 mM MgCl2, and 50 mM potassium phosphate, and was then centrifuged at 105,000 *g* for 90 min. The supernatant was dialyzed overnight against 20 mM potassium phosphate buffer (pH 7.4) and 1 mM 2-mercaptoethanol, and was then used as the source of crude TdR-Pase. The protein concentration was determined by the method of Lowry et al. [7]. TdR-Pase activity was calibrated against that measured using standard solutions, and is presented as unit/mg tissue protein. A 96-well microtiter plate (Nunc-immuno Plate Maxisorp; Nunc, Roskilde, Denmark) was incubated overnight at 4°C with 10 μg/ml of a TdR-Pase MoAb (104B) in 10 mM phosphate-buffered saline (PBS, pH 7.6). The plate was then coated with 3% (w/v) skim milk in PBS (blocking buffer) for 1 h at room temperature. The plate was washed with PBS containing 0.05% Tween 20 and 0.05% sodium azide and stored at 4°C until use. Test samples and standard solutions of TdR-Pase, which are HCT 116 tumor homogenates serially diluted with a blocking buffer, were dispensed onto the plate coated with antibody. The plate was: (1) incubated at 37°C for 1 h and then washed with 0.05% Tween20 in PBS, (2) incubated with MoAb 232–2 at 1 μg/ml in blocking buffer for 1 h at 37°C and washed, (3) incubated with 2,000-fold diluted anti-mouse IgG conjugated with horseradish peroxidase (Bio-Rad, Hercules, Calif.) for 30 min at 37°C and washed, and (4) incubated with a substrate solution containing 3,3′,5,5′-tetramethylbenzidine (TMB) and H_2O_2 (TMB microwell peroxidase substrate system, KPC, Gaithersburg, Md.) for 10–20 min at room temperature. The peroxidase reaction was stopped by the addition of 1 M phosphate solution, and the amount of TdR-Pase sandwiched with the two anti-TdR-Pase MoAb was estimated by measuring absorbency at 450 nm with a plate reader (Bio-Rad, model 3550).

In vitro histoculture drug response assay

In order to assess the antitumor effect of 5-FU and 5′-DFUR, an in vitro histoculture drug response assay (HDRA) was performed on cancer tissues obtained from 33 patients. Each drug was diluted in a complete medium that contained RPMI 1640 medium (Nissui, Tokyo, Japan) supplemented with 20% heat-inactivated fetal bovine serum, 2 mM L-glutamine, and antibiotics (100 units/ml penicillin and 100 μg/ml streptomycin) at ten times the therapeutic peak plasma concentration achieved by the administration of clinical doses. The concentration of 5-FU was 30 μg/ml and that of 5′-DFUR was 100 μg/ml. HDRA was performed as previously reported [3]. Cancer tissue was identified by the naked eye and cut into pieces approximately 2–3 mm in size. After weighing each piece, the pieces were placed on 1 cm^3 cubes of collagen sponge gel in the wells of a 24-well plate. Then each tumor piece was immersed in 800 μl of complete medium and 200 μl of an anticancer drug solution, followed by incubation at 37°C in a humidified 5% CO_2 atmosphere for 7 days. Each drug was assessed in triplicate. Three tumor pieces, incubated with 1,000 μl of complete medium without any anticancer drugs, were used as the control. After 7 days of incubation, 100 μl of RPMI 1640 medium containing 0.06% collagenase (type 1: Sigma) and 100 μl of 0.2% MTT (Sigma) solution were added to each well. The plate was then incubated for an additional 24 h. The supernatant in each well was aspirated carefully and 500 μl of dimethyl sulfoxide (DMSO, Wako Pure Chemicals, Osaka, Japan) was added to solubilize the MTT-formazan. After another 4 h of incubation, 100 μl of the MTT-formazan solution from each well was transferred to the wells of a 96-well microplate and the absorbance of each well was read with a microplate reader (Bio-Tek Instruments, USA) at a wave length of 550 nm. The inhibition rate (IR) was calculated as follows: IR(%) = (1–mean absorbance for drug-treated tumor/g/mean absorbance for control tumor/g)×100.

5′-DFUR was kindly provided by Nippon Roche (Tokyo, Japan), and 5-FU was purchased commercially. Collagen sponge gel was purchased from Yamanouchi Pharmaceutical (Tokyo, Japan).

Statistical analysis

Statistical analysis was performed using Student's *t*-test, the two-sample Wilcoxon signed-rank test, one-factor ANOVA, Scheffe's *F*-test, and Pearson's correlation. *P* values of < 0.05 were considered to be statistically significant.

Results

TdR-Pase activity in RCC and normal kidney tissue

As shown in Table 1, the TdR-Pase activity of RCC specimens was 13-fold that of normal kidney tissue. When the 39 pairs of specimens obtained from both tumor and normal tissue of the same kidney were compared, significant differences were clearly demonstrated. The TdR-Pase activity of the tumor specimens was 14-fold that of the normal specimens (tumor tissue vs normal kidney tissue: 205.0 ± 34.7 vs 14.9 ± 1.6 unit/mg protein, Wilcoxon's signed-rank test: $P < 0.0001$).

TdR-Pase activity and histological parameters

As the histological grade increased, so the TdR-Pase activity of RCC specimens also increased (Table 2). The TdR-Pase activity of G 1 RCC specimens was significantly lower than that of G 2 and G 3 specimens. An increase of TdR-Pase activity was also observed with an increase in tumor histological stage. When the RCCs

Table 1. Comparison of thymidine phosphorylase (TdR-Pase) activity between (renal cell carcinoma) RCC and normal kidney tissue (significance: $P < 0.0001$). Results are shown as mean ± SE

Specimens	*n*	TdR-Pase activity (unit/mg protein)
RCC	61	191.6 ± 24.7
Normal	39	14.9 ± 1.6

Table 2. TdR-Pase activity and histological grade. Results are shown as the mean ± SE. *,** $P < 0.05$ versus Gl by Fisher's protected least significant difference

Histological grade	*n*	TdR-Pase activity (unit/mg protein)
Grade 1	16	96.1 ± 16.9
Grade 2	40	212.3 ± 32.6*
Grade 3	5	331.4 ± 111.1**

were divided into low-stage tumors (T2 or lower) and high-stage tumors (T3 or higher), the TdR-Pase activity of the high-stage tumors was found to be significantly greater than that of the low-stage tumors (Table 3).

TdR-Pase activity and chemosensitivity to FU-related drugs

If TdR-Pase activity affects the chemosensitivity of cancer cells to FU-related drugs, then tumor cells with high TdR-Pase activity should be more sensitive to these drugs. Table 4 shows the relationship between the histological grade and sensitivity to 5′-DFUR/5-FU as determined by HDRA. As the histological grade increased, so the inhibition rate (IR) for 5′-DFUR/5-FU increased (Table 4). The IR for 5-FU of G 3 tumors was significantly higher than that of G 1 and G 2 tumors (Table 4). In addition, TdR-Pase activity exhibited a significant positive correlation with IR for 5′-DFUR and 5-FU (Fig. 1). Furthermore, there was a significant positive correlation between the IR values for 5-FU and 5′-DFUR ($r = 0.516$, $P = 0.003$), suggesting that HDRA had been performed correctly.

Table 3. TdR-Pase activity and histological stage (significance: $P < 0.05$). Results are shown as the mean ± SE

Histological stage	*n*	TdR-Pase activitv (unit/mg protein)
≤ T2	47	164.3 ± 21.9
T3 ≤	14	283.1 ± 75.9*

Table 4. Relationship between sensitivity to FU-related drugs and histological grade of RCC. Results are shown as the mean ± SE. *$P < 0.05$ versus Grade 3 analyzed by Scheffe's F

Histological Grade	*n*	Inhibition Rate	
		5-FU	5'-DFUR
Grade 1	9	22.2 ± 8.3*	9.6 ± 3.1
Grade 2	21	26.8 ± 5.0*	14.9 ± 3.4
Grade 3	3	68.6 ± 9.2	31.1 ± 7.8

Discussion

Our results confirmed the findings of a previous study that the TdR-Pase activity of tumor tissues was significantly higher than that of normal kidney tissues and that RCCs with high TdR-Pase activity may be high-grade and high-stage cancers [5].

TdR-Pase is a key enzyme in FU metabolism, being essential for the initial process of 5-FU activation and also the conversion of 5′-DFUR to 5-FU. Theoretically, FU-related drugs should be effective against high TdR-Pase RCCs. Morita et al., in their fundamental investigation, confirmed the findings that the sensitivity of human renal carcinoma cells to capecitabine, a novel fluoropyrimidine carbamate, was enhanced by transfection of TdR-Pase cDNA [10]. In Order to investigate the potential clinical application of individualized FU therapy based on the TdR-Pase of RCC tissue, we utilized surgically obtained tissues and studied the correlation between TdR-Pase activity and chemosensitivity to FU-related drugs by HDRA. Our findings revealed a significant positive correlation between TdR-Pase activity and the cytotoxic effect of FU-related drugs. Because RCC is generally thought to be resistant to chemotherapy, immune therapy with agents such as interferons and/or interleukins has been used for treatment. However the clinical results have not been satisfactory. The present study suggests that FU therapy might be a strategy for RCCs with high TdR-Pase activity. Furthermore, in combination with interferon α, which is

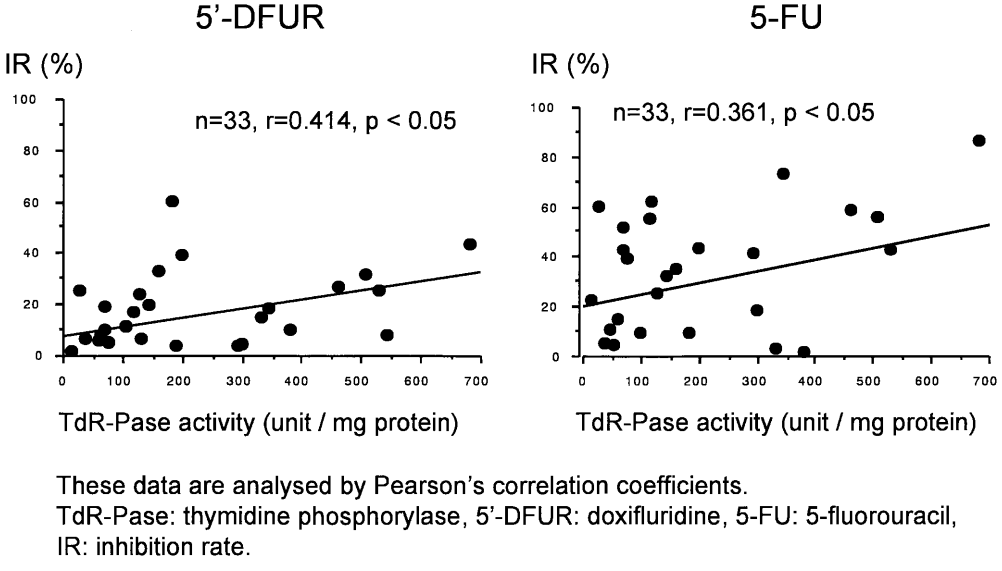

Fig. 1. Correlation between thymidine phosphorylase (*TdR-Pase*) activity and chemosensitivity to doxifluridine (*5′-DFUR*)/5-fluorouracil (*5-FU*). These data were analysed by Pearson's correlation coefficient. *IR* indicates inhibition rate

Table 5. Comparison of TdR-Pase activity and sensitivity between RCC and TCC. [3]. Results are shown as the mean ± SE

	RCC	*n*	TCC*	*n*	*P*
TdR-Pase activity (unit/mg protein)	191.6 ± 24.7	61	65.0 ± 10.9	62	< 0.0001
Inhibition rate to 5-FU(%)	29.5 ± 4.6	33	56.2 ± 4.5	33	= 0.0001
Inhibition rate to 5′-DFUR(%)	14.9 ± 2.6	33	33.4 ± 4.7	33	= 0.0009

known to up-regulate TdR-Pase expression [11, 13], it might enhance the therapeutic benefit. Although there was a significant positive correlation between TdR-Pase activity and cytotoxicity, the mean inhibition rate (IR) of 5-FU/5′-DFUR obtained with our HDRA was still less than 50% except for G3 specimens. 5-FU/5′-DFUR administration alone thus appeared inadequate for obtaining an acceptable cytotoxic effect against RCC. We previously reported the relationship between TdR-Pase activity and chemosensitivity of transitional cell carcinoma [4]. As shown in Table 5, when TdR-Pase activity and the sensitivity of RCC and TCC specimens to 5-FU/5′-DFUR were compared, TdR-Pase activity of RCC specimens was found to be significantly higher than that of TCC specimens. However, the mean IR to FU-related drugs of RCCs was significantly lower than that of TCCs. These results suggest that not only TdR-Pase, but also other factors such as thymidilate synthase (TS) and dihydropyrimidine dehydrogenase (DPD) might influence the 5-FU sensitivity of RCC. 5-FU inhibits TS, which is the key enzyme in the catalysis of the methylation from deoxyuridine monophosphate to deoxythymidine monophosphate, and leads to the blocking of DNA synthesis. On the other hand, 5-FU is degraded through the catabolic pathway with DPD. Recently, TS and DPD activity have been measured in clinically obtained cancer tissues such as gastric cancers, colorectal cancers, and neck cancers, [1, 8, 9, 12, 16] and some investigators have reported that high-TS and/or high-DPD activity in cancer tissue was involved with 5-FU resistance [1, 8, 9, 12, 16]. Because such a relationship has not been clarified in RCC, we are continuing to investigate the association between TdR-Pase, TS and DPD activity and 5-FU sensitivity in RCC.

In conclusion, TdR-Pase is an independent parameter for predicting the malignant potential of RCC, and might also be a good predictive factor for sensitivity to FU-related drugs. However, further studies are needed to overcome low sensitivity of 5-FU in RCC.

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