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ORIGINAL ARTICLE

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# Clinical significance of thymidine phosphorylase and dihydropyrimidine dehydrogenase expression in transitional cell cancer

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**Abstract** *Purpose*: Thymidine phosphorylase (TdR-Pase) and dihydropyrimidine dehydrogenase (DPD) are thought to be key enzymes in the metabolic pathways of 5-fluorouracil (5-FU). Theoretically, tumors which have low DPD and/or high TdR-Pase expression should be 5-FU-sensitive. TdR-Pase also has angiogenic expression which aids tumor progression and metastasis. However, little is known concerning the relationship between DPD expression and clinical malignant potential, especially in urological cancer. *Materials and methods*: Transitional cell cancer (TCC) tissues were obtained from 50 patients, and TdR-Pase and DPD expression was measured by ELISA and radioenzyme assay, respectively. The sensitivity of 23 of the 50 specimens to 5-FU was assessed in a histoculture drug response assay (HDRA), an in vitro chemosensitivity test. *Results*: TdR-Pase and DPD expression in TCC tissues was higher than in normal urothelial tissues. The expression of both TdR-Pase and DPD in TCC increased with histological grade and stage. Superficial bladder cancer patients who had undergone transurethral resection were divided into two groups, a recurrent and a nonrecurrent group. The expression of TdR-Pase and DPD was higher in the recurrent group than in the nonrecurrent group, but the differences were not significant. There was a significant inverse correlation between DPD expression and 5-FU sensitivity. However, TdR-Pase exhibited no correlation with 5-FU sensitivity. *Conclusions*: The expressions of both enzymes may be a good indicator of the malignant potential of TCC. Although DPD may be a good indicator of sensitivity of TCC to 5-FU, TdR-Pase appeared not to regulate the sensitivity of TCC to 5-FU.

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## Introduction

5-Fluorouracil (5-FU) is widely used in the treatment of various cancers including urothelial cancers [11]. It is metabolized by various enzymes in vivo and is finally converted to 5-fluoro-2′-deoxyuridine 5′-monophosphate (FdUMP), which binds with thymidylate synthase (TS) and $N_5N_{10}$ methylene tetrahydrofolate (Me-THF), thereby blocking DNA synthesis [4]. 5-FU is also converted to 5-fluorouridine 5′-triphosphate (FUTP), which interferes with RNA synthesis [4].

Thymidine phosphorylase (TdR-Pase) is one of the key enzymes in the metabolism of 5-FU, since it is necessary for the initial conversion of 5-FU to 5-fluoro-2′-deoxyuridine (FdUrd) [11]. TdR-Pase is also known to be identical to platelet-derived endothelial cell growth factor (PDECGF), which has potent angiogenic activity [9]. High levels of activity of this enzyme have been observed in various malignant tumors [8, 13, 16]. A correlation between the activity of this enzyme and the malignant potential of tumors has also been observed [8, 13, 16].

5-FU is catabolized to 2-fluoro-$\beta$-alanine by three enzymes: dihydropyrimidine dehydrogenase (DPD), followed by dihydropyrimidinase and $\beta$-ureidopropionase. Of these three enzymes, DPD is the first and rate-limiting enzyme. DPD expression is high in the liver and peripheral blood mononuclear cells, and is also high in cancer cells. Recently, DPD expression has been measured in cancer tissues obtained clinically such as gastric cancers, colorectal cancers, and neck cancers, and the significance of DPD expression in cancer cells as an indicator of malignant potential has been demonstrated [1, 14, 24]. Considering the impact of TdR-Pase and DPD in cancer cells on the effectiveness of 5-FU, theoretically, cancer cells which have high TdR-Pase expression and/

or low DPD expression should be sensitive to 5-FU by virtue of their ability to maintain a high concentration of 5-FU for a long time. Relationships between the two enzymes and the effectiveness of 5-FU against cancers have recently been reported [10, 20]. However, little is known concerning such a relationship in urological cancer.

In this study, we measured the expression of both TdR-Pase and DPD in cancer tissues obtained surgically, and investigated the relationship between these enzymatic activities and histological parameters. In addition, the results of in vitro chemosensitivity testing were analyzed to determine whether TdR-Pase and/or DPD expression in cancer cells can predict the efficacy of 5-FU.

## Materials and methods

### Cancer specimens

Between July 1996 and May 2000, 50 transitional cell cancer (TCC) tissue specimens were obtained during either total cystectomy or transurethral cold-cup removal at Hamamatsu University Hospital and related hospitals. Informed consent to study TdR-Pase expression, DPD expression, and chemosensitivity was obtained from all patients before surgery. Of the 50 TCC specimens, 38 were from the bladder, 9 were from the renal pelvis, and 3 from the ureter. All the cancer tissue specimens were histologically confirmed to be TCC. The histological grade and stage according to the TNM classification were as follows: grade 1 $n=8$, grade 2 $n=26$, and grade 3 $n=16$; Ta $n=4$, T1 $n=17$, T2 $n=5$, T3 $n=15$, and T4 $n=9$.

### Normal bladder tissue

From seven cystectomy specimens, samples of normal bladder mucosa were obtained. An area far from the TCC was selected and 1 $cm^2$ of the mucosal layer was removed. The tissues were confirmed to be TCC and normal bladder tissue by a pathologist.

### Enzyme-linked immunosorbent assay (ELISA) for TdR-Pase expression

Specimens were rapidly frozen and stored at –80ºC until assay. The tissue was homogenized in 10 m*M* Tris-HCl buffer (pH 7.4) containing 15 m*M* NaCl, 1.5 m*M* $MgCl_2$, and 50 m*M* potassium phosphate, and then was centrifuged at 105,000 *g* for 90 min. The supernatant was dialyzed overnight against 20 m*M* potassium phosphate buffer (pH 7.4) and 1 m*M* 2-mercaptoethanol, and was then used as the source of crude TdR-Pase. Protein concentration was determined by the method of Lowry et al. [12]. TdR-Pase expression was calibrated against that of standard solutions, and is presented as units per milligram tissue protein. A 96-well microtiter plate (Nunc-immuno Plate Maxisorp; Nunc, Roskilde, Denmark) was incubated overnight at 4ºC with 10 μg/ml of a TdR-Pase mAb (104B) in 10 m*M* phosphate-buffered saline (PBS, pH 7.6). Then the plate was coated with 3% (w/v) skimmed 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 4ºC until use. Test samples and standard solutions of TdR-Pase (HCT 116 tumor homogenates serially diluted with a blocking buffer) were dispensed onto the plate coated with antibody. The plate was subjected to four incubation steps: (1) at 37ºC for 1 h and then washed with 0.05% Tween 20 in PBS; (2) with mAb 232-2 at 1 μg/ml in blocking buffer for 1 h at 37ºC and washed; (3) with 2000-fold diluted anti-mouse IgG conjugated with horseradish peroxidase (Bio-Rad, Hercules, Calif.) for 30 min at 37ºC and washed; and (4) with a substrate solution containing 3,3′,5,5′-tetramethylbenzidine (TMB) and $H_2O_2$ (TMB Microwell Peroxidase Substrate System; KPL, 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 mAbs was estimated by measuring absorbency at 450 nm with a plate reader (Bio-Rad, model 3550).

### Enzymatic assay for DPD expression

The DPD enzymatic assay was based on the method described previously by Naguib et al. [18]. DPD expression was determined by measuring the sum of the products, dihydrofluorouracil (DHFU), 2-fluoro-β-ureidopropionate (F-β-UPA), and F-β-Ala, formed from [6-$^{14}$C]5-FU. The standard mixture contained 10 m*M* potassium phosphate (pH 8.0) containing 0.5 m*M* EDTA and 0.5 m*M* β-mercaptoethanol, 2 m*M* dithiothreitol, 5 m*M* $MgCl_2$, 20 μ*M* [6-$^{14}$C]5-FU, 100 μ*M* NADPH, and 25 μl cytosol in a final volume of 50 μl. Incubations were carried out at 37ºC for 30 min. The reaction was terminated by immersing the reaction tubes in a bath of boiling water for 2 min. 5-FU and the products were isolated by thin-layer chromatography (TLC) according to the method of Ikenaka et al. [7]. The mixture after the reaction was added to 25 μl 0.36 m*M* KOH, left to stand at room temperature for at least 30 min to hydrolyze the DHFU formed, and then mixed with 25 μl 0.36 m*M* $HClO_4$ for neutralization and centrifuged at 14,000 rpm for 5 min. A 5-μl aliquot of the supernatant was applied to a TLC plate (silica gel 60 F254; Merck, Germany), and developed with a mixture of ethanol and 1 *M* ammonium acetate (5:1, v/v). Each product was visualized and quantified using an image analyzer (BAS-2000; Fujix, Tokyo, Japan).

### In vitro histoculture drug response assay

A sufficient volume of cancer tissue was obtained from 23 patients to perform an in vitro histoculture drug response assay (HDRA) to assess the antitumor effect of 5-FU. 5-FU was diluted in complete medium comprising RPMI-1640 medium (Nissui, Tokyo, Japan) supplemented with 20% heat-inactivated fetal bovine serum, 2 m*M* L-glutamine, and antibiotics (100 U/ml of penicillin and 100 μg/ml of streptomycin). The final concentration of 5-FU used in the HDRA was 30 μg/ml, ten times the therapeutic peak plasma concentration achieved by intravenous administration of clinical doses. The HDRA was performed as previously described [5]. Cancer tissue was identified visually and cut into pieces approximately 2–3 mm in size. After weighing, each piece was placed on a 1-cm cube of collagen sponge gel in the wells of a 24-well plate. Then each tumor piece was immersed in 800 μl complete medium and 200 μl of an anticancer drug solution, followed by incubation at 37ºC in a humidified atmosphere containing 5% $CO_2$ for 7 days. Triplicate samples were evaluated. Three tumor pieces incubated with 1000 μl complete medium without any anticancer drug were used as the control.

After 7 days of incubation, 100 μl RPMI-1640 medium containing 0.06% collagenase (type 1, Sigma) and 100 μl 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 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, Santa Barbara, Calif.) at a wavelength of 550 nm. The inhibition rate (IR) was calculated as follows: IR(%) = (1–mean absorbance per gram drug-treated tumor/mean absorbance per gram control tumor)×100. 5-FU and collagen sponge gel were purchased from Kyowa Hakko Pharmaceutical Company (Tokyo, Japan) and Yamanouchi Pharmaceutical Company (Tokyo, Japan), respectively.

### Statistical analysis

Statistical analysis was performed using the Mann-Whitney *U*-test, one-factor ANOVA, and Fischer's protected least significant difference test, and calculation of Pearson's correlation coefficients. *P* values $< 0.05$ were considered statistically significant.

## Results

### TdR-Pase and DPD expression in TCC and normal bladder mucosa

Figure 1 shows comparisons of TdR-Pase and DPD expression between TCC specimens and normal bladder tissues. TdR-Pase expression in TCC specimens was fourfold that in normal bladder tissue, and DPD expression in TCC specimens was twofold that in normal tissue. There were no differences in the expression of these two enzymes between renal pelvic and ureteral tumors and bladder tumors (Table 1).

### TdR-Pase and DPD expression and histological parameters

Figures 2 and 3 show the relationships between TdR-Pase and DPD expression and histological parameters. As histological grade increased, TdR-Pase expression in TCC specimens also increased (Fig. 2). The TdR-Pase expression in grade 3 TCC specimens was significantly higher than in grade 1 and 2 specimens. The DPD expression in TCC specimens also increased with histological grade (Fig. 2). The DPD expression in grade 3 TCC specimens was significantly higher than in grade 1 specimens. This tendency was found even after tumors were subclassified into pelvis/ureter cancer and bladder cancer (Table 2). An increase in TdR-Pase and DPD expression was also observed with increase in tumor histological stage: the expression of TdR-Pase and DPD in invasive (T2 to T4) tumors was found to be significantly higher than in superficial (Ta plus T1) tumors (Fig. 3).

**Table 1** Comparison of TdR-Pase and DPD activities between tumors from different sites. There were no significant differences between tumors from different sites as determined by the Mann-Whitney *U*-test. The results are shown as means ± SE

| Tumor location | TdR-Pase activity (U/mg protein) | DPD activity (pmol/mg protein/min) |
|---|---|---|
| Pelvis and ureter ($n = 12$) | 102.1 ± 45.4 | 107.9 ± 23.7 |
| Bladder ($n = 38$) | 75.1 ± 10.8 | 103.9 ± 15.2 |

### Comparison of TdR-Pase and DPD expression between superficial bladder cancer patients with postoperative recurrence and those with nonrecurrence

Of the 50 patients, 14 with superficial bladder cancer had undergone transurethral resection alone without any adjuvant chemotherapy. The mean follow-up period of these patients was 30 months. They were divided into two groups, a recurrent and a nonrecurrent group. Of the 14 patients, 6 belonged to the recurrent group. Recurrence was noted between 7 and 27 months after surgery. TdR-Pase expression in the recurrent group was higher than in the nonrecurrent group (69.8 ± 32.3 vs 38.7 ± 17.4 U/mg protein). Additionally, DPD expression was also slightly higher in the recurrent group than in the nonrecurrent group (76.4 ± 24.5 vs 59.8 ± 14.0 pmol/mg protein per min). These differences, however, were not significant.

### Relationship between TdR-Pase and DPD expression and sensitivity to 5-FU

If TdR-Pase and DPD in cancer cells influence sensitivity to 5-FU, cancer cells with high TdR-Pase and/or low DPD expression might be more sensitive to 5-FU. We compared the expressions of these two enzymes with 5-FU sensitivity determined in the HDRA. There was a significant inverse correlation between DPD expression and inhibition rate (IR%, Fig. 4). However, 5-FU sensitivity exhibited no correlation with TdR-Pase expres-

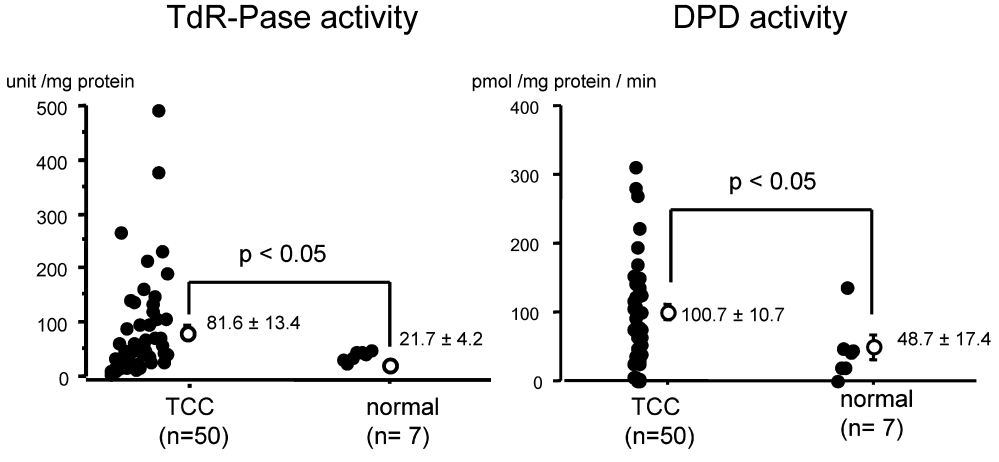


**Fig. 1** Comparison of TdR-Pase and DPD expression between TCC specimens and normal urothelial tissue specimens. TdR-Pase expression in TCC specimens was fourfold higher than in normal bladder tissue, and DPD expression in TCC specimens was also twofold higher than in normal tissue. The results are shown as the means ± SE. Statistical analysis was carried out using the Mann-Whitney *U*-test

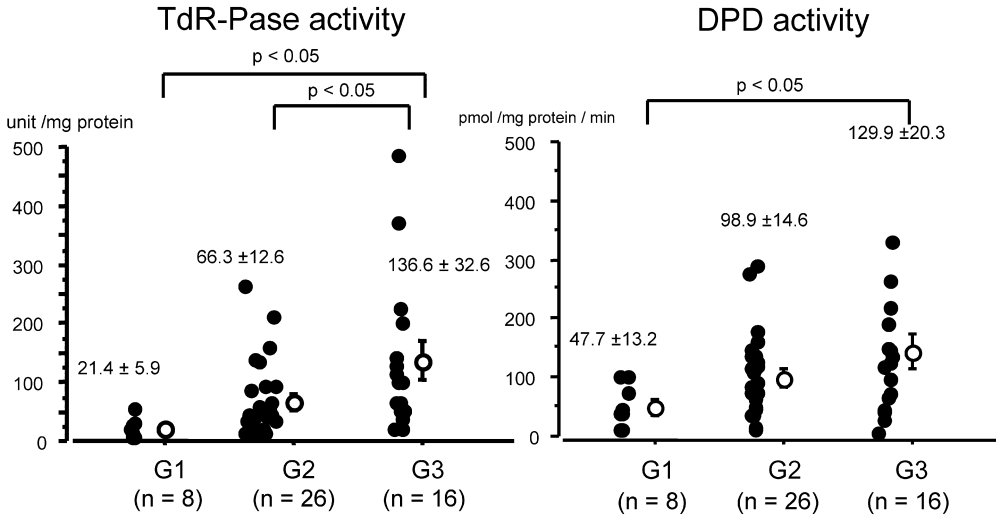


**Fig. 2** TdR-Pase and DPD expression and histological grade. As the histological grade increased, the TdR-Pase expression in TCC specimens also increased. TdR-Pase expression in grade 3 TCC specimens was significantly higher than that of grade 1 and 2 specimens. The DPD expression in TCC specimens also increased with histological grade. The DPD expression in grade 3 TCC specimens was significantly higher than in grade 1 specimens. The results are shown as the means ± SE. Statistical analysis was carried out using Fischer's protected least significant difference test (*G* histological grade)

sion. Of the 23 specimens assessed for 5-FU sensitivity by HDRA, 13 (56.5%) were classified as high sensitivity (IR ≥50%) and 10 (43.5%) as low sensitivity (IR < 50%). DPD expression was significantly lower in the high-sensitivity group than in the low-sensitivity group (Table 3). However, TdR-Pase expression did not differ between the two groups (Table 3). Table 4 shows a comparison of sensitivity to 5-FU by histological grade and stage. The IRs of histological grade 1 specimens were significantly higher than those of high-grade specimens (grade 2/3). In addition, the IRs of superficial (Ta plus T1) TCC specimens were significantly higher than those of invasive (T2–T4) specimens. These results suggest that TCCs with high malignant potential are resistant to 5-FU therapy because of high DPD expression.

## Discussion

Despite the use of fluorouracil-related drugs for the treatment of urothelial cancer [11], little is known concerning the relationship among histological parameters, 5-FU sensitivity, and DPD and TdR-Pase expression. Concerning DPD in TCC, in particular, there have been no reports since that by Mizutani et al. [17]. Although the present study was very small, our findings may support their results.

The findings for TdR-Pase expression confirmed those of our previous report [6]. That is, TdR-Pase expression in TCCs increased with histological grade and stage, but no correlation was found between TdR-Pase expression and 5-FU sensitivity. Given these results, we considered it necessary to investigate other possibly important metabolic or catabolic enzymes for 5-FU which regulate the sensitivity of TCCs to 5-FU. Some investigators believe that 5-FU-related drugs might be effective for high-TdR-Pase TCC because TdR-Pase is required for the first stage of 5-FU activation [21]. 5-FU, however, can be metabolized in vivo in any of the following three ways. The first is by the conversion of 5-FU

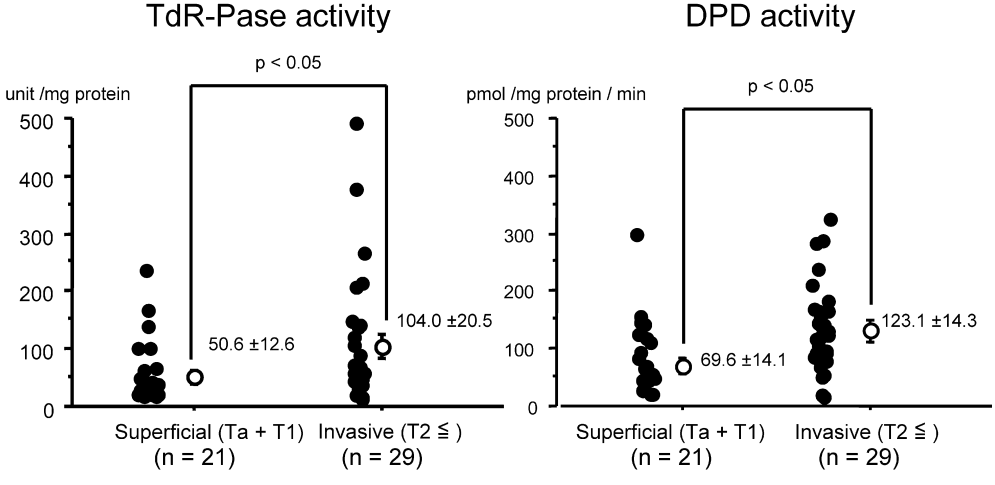


**Fig. 3** TdR-Pase and DPD expression and histological stage. TdR-Pase and DPD expression also increased with histological stage. TdR-Pase and DPD expression in invasive tumors (T2–T4) was significantly higher than in superficial tumors (Ta plus T1). The results are shown as the means ± SE. Statistical analysis was carried out using the Mann-Whitney *U*-test

**Table 2** TdR-Pase and DPD activities and histological grade in pelvic and ureteral cancer and bladder cancer. The results are shown as means ± SE

| Tumor location | Grade | TdR-Pase activity (U/mg protein) | DPD activity (pmol/mg protein/min) |
|---|---|---|---|
| Pelvis and ureter | 1 ($n=2$) | 10.2 ± 4.4 | 86.8 ± 6.9 |
| | 2 ($n=8$) | 43.5 ± 11.6 | 92.8 ± 31.4 |
| | 3 ($n=2$) | 428.8 ± 59.2* | 189.3 ± 36.6 |
| Bladder | 1 ($n=6$) | 25.1 ± 7.2 | 34.7 ± 13.6 |
| | 2 ($n=18$) | 76.4 ± 17.1 | 101.6 ± 16.4 |
| | 3 ($n=14$) | 94.8 ± 16.9** | 136.4 ± 32.5** |

*$P < 0.05$ vs grades 1 and 2, **$P < 0.05$ vs grade 1, Fischer's protected least squares difference

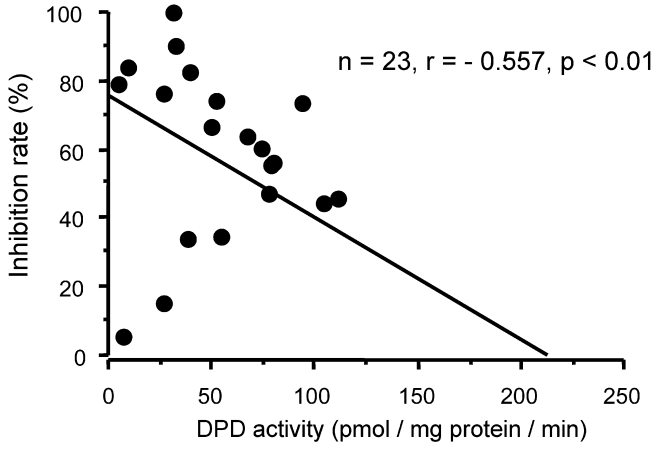


**Fig. 4** Correlation between DPD expression and 5-FU sensitivity. There was a significant inverse correlation between DPD expression and inhibition rate (IR%) of 5-FU. Statistical analysis was carried out by calculation of Pearson's correlation coefficient

to 5-fluorouridine 5′-monophosphate (FUMP) by orotate phosphoribosyl transferase (OPRT). The second is by conversion of 5-FU to FUMP by uracil ribosyltransferase plus uridine kinase. The third is conversion of 5-FU to FdUrd by TdR-Pase. It is not clear which route is the principal one, because differences exist among individuals, even those with the same type of cancer. However, basic investigations have indicated that the first and second routes may be the principal ones [2, 22]. Furthermore, the possibility of opposing conversion from FdUrd to 5-FU by TdR-Pase has also been reported [25]. These findings suggest reasons why TdR-Pase cannot play a role in regulating 5-FU sensitivity in TCC.

**Table 4** Relationships between 5-FU sensitivity and histological parameters

| | Inhibition rate (%) |
|---|---|
| Histological grade | |
| Low (grade 1, $n=6$) | 80.3 ± 6.1* |
| High (grades 2 + 3, $n=17$) | 41.6 ± 6.9 |
| Histological stage | |
| Superficial (Ta + T1, $n=11$) | 70.9 ± 7.0** |
| Invasive (T2–T4, $n=12$) | 34.2 ± 7.7 |

*$P < 0.01$ vs high grade, **$P < 0.01$ vs invasive, Student's $t$-test

In contrast to the findings for TdR-Pase, the present study suggests that DPD may regulate 5-FU sensitivity of TCC and may also be a good indicator of the malignant potential of TCC. DPD expression in TCC tissues was widely distributed from 2.6 to 312.8 pmol/mg protein per min (mean 100.6 pmol/mg). The DPD expression reported in cancers of other organs are as follows (pmol/mg protein per min): colorectal cancers 27.9–206.9 [14], metastatic liver cancers 87.9–219.6 [14], and head and neck cancers 13–193 [1]. The present study demonstrated that DPD expression in TCC increases with histological grade. Additionally, among superficial bladder cancer patients who had undergone transurethral resection, DPD expression was higher in the recurrent group than in the nonrecurrent group, although not to a significant extent. These results suggest that DPD, as in other organ cancers [1, 3, 14, 24], may be an important indicator for predicting the malignant potential of TCC.

In the present study, DPD expression in TCCs significantly differed from that in normal bladder tissues. The tumor DPD/normal DPD ratios of seven paired specimens were calculated. The mean ratio was found to be 2.52 (range 1.05–5.50). In addition, three of the seven specimens (43%) had a ratio greater than 2. In head and neck cancers, the mean ratio has been found to be 1.04 (range 0.26 –6.6), and 7 of 42 tumors (17%) had a ratio greater than 2 [1]. McLeod et al. found colorectal cancers to have a mean ratio of 0.76 (ranged 0.19–3.32), and 3 of 63 tumors (5%) had a ratio greater than 2 [14]. Guimbaud et al. found colorectal cancers to have a mean ratio of 0.97 (range 0.19–3.32) and 9 of 70 (13%) had a ratio greater than 2 [3]. The mean ratio in the present study was higher than those for other organs. This finding may be due to the small number of paired specimens (only seven cystectomied patients).

In addition, all the paired specimens were histologically diagnosed as high-grade and/or high-stage TCCs.

**Table 3** Comparison of high-sensitivity and low-sensitivity groups. The results are shown as means ± SE

| | High-sensitivity group ($n=13$) | Low-sensitivity group ($n=10$) |
|---|---|---|
| Inhibition rate (%) | 74.1 ± 3.7* | 22.7 ± 6.5 |
| TdR-Pase activity (U/mg protein) | 56.8 ± 18.1 | 55.1 ± 14.9 |
| DPD activity (pmol/mg protein/min) | 49.2 ± 7.8 | 89.7 ± 19.1** |

*$P < 0.0001$ vs low-sensitivity group, **$P < 0.05$ vs high-sensitivity group, Student's $t$-test

Further investigations using many paired specimens including low-grade/low-stage TCCs appear to be necessary, because the fact that high-grade/high-stage carcinomas have a higher ratio of tumor to stroma than low-grade/low-stage tumors should be taken in to account. However, Mizutani et al., who investigated DPD expression in TCC using 82 specimens including various grades and stages, also reported that DPD expression in bladder cancer tissue is twice that in normal bladder tissue [17]. This may be a reason why single-agent 5-FU is not effective for TCC. The present study revealed a significant inverse correlation between DPD expression and 5-FU sensitivity in TCC tissues. Additionally, the present study revealed that many invasive TCC specimens belonged to the low-sensitivity group, suggesting that high-grade and high-stage TCCs have high levels of DPD expression and that treatment with 5-FU cannot be expected to be effective against such TCCs. However, low-grade and/or superficial TCCs exhibited high sensitivity to 5-FU. In addition to this finding, all the TCC specimens with high 5-FU sensitivity (IR ≥50% ) exhibited less than 100 pmol/mg protein per min of DPD expression.

DPD thus appears to be an important factor regulating 5-FU sensitivity in TCC. Enhancement of sensitivity to 5-FU with the use of DPD inhibitors including interferon [15], eniluracil [2], 5-chloro-2,4-dihydroxypyridine (CDHP) [22, 23] has been reported in basic investigations. In addition, good clinical results with S-1, a new oral FU drug which consists of tegafur, CDHP and potassium oxonate in a molar ratio of 1:0.4:1 [23], have recently been reported [19]. Enhancement of sensitivity to 5-FU with the use of DPD inhibitors may also be useful for TCC.

In conclusion, the current study demonstrated that both TdR-Pase and DPD expression in TCC increased with tumor histological grade and stage, and that elevated levels of TdR-Pase and DPD expression may be associated with recurrence of superficial bladder cancer. Both enzymes appear to be predictive indicators of the malignant potential of TCC. Although further investigations of factors regulating 5-FU sensitivity in TCC such as OPRT and thymidylate synthase appear to be necessary, DPD expression in TCC may be one such factor.

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