Significance of 5-Fluorouracil-related Enzyme Activities in Predicting Sensitivity to 5-Fluorouracil in Bladder Carcinoma

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Abstract. Background: The association between 5-fluorouracil (5-FU)-related enzyme activity and the sensitivity of bladder urothelial carcinoma (BUC) to 5-FU were investigated, and methods to improve 5-FU sensitivity were analyzed. Materials and Methods: Tumor specimens were obtained from 127 patients. Orotate phosphoribosyl transferase (OPRT) activity was analyzed by the paper disc method and thymidine phosphorylase and dihydropyrimidine dehydrogenase (DPD) activities were measured by ELISA. 5-FU sensitivity was assessed in 99 cases by an in vitro chemosensitivity test. Results: A significant positive correlation between OPRT activity level and the sensitivity of BUC to 5-FU was identified. Moreover, the combination of 5-FU and 5-chloro-2,4-dihydroxypyrimidine significantly enhanced 5-FU sensitivity in BUC, particularly in cases showing higher DPD activity. Conclusion: OPRT was the most important enzyme in predicting sensitivity to 5-FU in BUC. These results may have implications for tailor-made medication using 5-FU-related compounds as postoperative adjuvant chemotherapy in BUC patients.

In bladder urothelial carcinoma (BUC), cisplatin (CDDP)-based combination regimens (e.g. MVAC or GC) (1) are commonly used in the treatment of muscle invasive carcinoma, whereas bladder instillation therapy with Bacillus Calmette-Guerin (2) or anticancer agents (3) are usually employed for non-muscle invasive tumors. 5-Fluorouracil (5-FU) is an anticancer agent in wide use against various other malignancies, however its use is less common in the treatment of BUC. Nevertheless, a comparatively large number of cases in Japan receive 5-FU-related compounds as postoperative adjuvant chemotherapy to prevent the recurrence of superficial or invasive BUC after curative surgery, particularly patients who appear unable to tolerate the side effects of intravesical instillation therapy or CDDP-containing combination chemotherapy. In this regard, a randomized prospective trial of long-term administration of UFT, a compound containing uracil and tegafur (a prodrug of 5-FU) in a 4:1 molar ratio, showed prophylactic efficacy in preventing recurrence with low toxicity after transurethral resection for superficial BUC (4). Nevertheless, the question of which BUC patients benefit from adjuvant treatment with 5-FU has not been investigated.

The use of 5-FU-related enzyme expression as a predictor of 5-FU efficacy against other malignancies has been widely investigated (5, 6). Studies focused on enhancing the antitumor activity of 5-FU have used methodologies such as the calculation of expression ratio of respective enzymes (7) or 5-FU modulators such as 5-chloro-2,4-dihydroxypyrimidine (CDHP), a dihydropyrimidine dehydrogenase (DPD) inhibitor (8). In advanced gastric carcinoma, for example, the clinical effectiveness of S-1, a new oral 5-FU-related drug consisting of tegafur, CDHP and potassium oxonate in a molar ratio of 1:0.4:1 (9), has been demonstrated in both postoperative adjuvant (10) and definitive (11) treatment settings. With regard to the management of BUC, however, these issues have received little attention.

In this report the usefulness of determining 5-FU-related enzyme activities in BUC specimens was evaluated in order to predict the sensitivity of patients with BUC to 5-FU. In addition, the value of individual enzyme activity ratios and modulation of 5-FU using CDHP to enhance the sensitivity of BUC to 5-FU in experimental settings was also investigated.

Materials and Methods

Tumor samples. Tumor specimens were obtained from 127 consecutive patients undergoing either total cystectomy or transurethral cold-cup removal for BUC at Hamamatsu University...
Hospital and affiliated hospitals between June 1996 and July 2004. They included 101 male and 26 female patients, ranging in age from 36 to 86 years. Written informed consent was obtained from all patients and the study was approved by the local Ethics Committee of each participating institution. All patients were histologically diagnosed as having urothelial carcinoma (UC). Histological grading and staging according to the 1997 TNM system are presented in Table I. Enzyme activity measurement in tumor tissues for OPRT, TdR-Pase and DPD was made in 54, 102 and 43 cases, respectively. Specimens were stored frozen at –80°C until use for assay.

Normal bladder samples. Normal bladder samples were obtained from 42 patients who underwent total cystectomy. An area remote from the UC lesion was selected and a section of mucosal layer measuring 1 cm² was removed. The tissues were confirmed to be normal bladder epithelium by reference to the case report of the local pathologist who originally diagnosed the case. Enzyme activity of OPRT, TdR-Pase and DPD in these normal bladder tissues was determined for 19, 39 and 27 cases, respectively (Table I). Samples were stored frozen at –80°C until assay.

Measurement of OPRT expression. OPRT activity was determined using the paper disc method (12). Briefly, BUC specimens and normal bladder tissues were homogenized and centrifuged at 105,000 g at 4°C for 1 h, and the supernatant was collected. A solution containing 50 μL [400mM MgCl₂]-400 mM Tris-HCl (pH 7.5), 50 μL 40 mM 5-phosphoribosyl-1-pyrophosphate (PRPP), 25 μL 240 mM 2-glycerophosphate, 25 μL 9.6 mM α,β-methylene adenosine 5’-diphosphate, 25 μL 63,993 mM [COLD]-5FU and 25 μL 0.1 mCi/ml 0.007 mM-[6-3H]-5FU in a total volume of 200 μL was added to 200 μL of the supernatant maintained and incubated at 37°C. At 5, 10 and 15 min after initiating incubation, 90 μL of the reaction solution was pipetted into a new tube and heated for 3 min in boiling water to stop the enzyme reaction. The reaction mixture, which formed a precipitate on heating, was centrifuged to separate the supernatant at 15000 g at 4°C for 30 min. A 25-μL portion of the supernatant was spotted onto a filter paper disc of DEAE-cellulose, and the disc was repeatedly washed to remove nonreacted [6-3H]-5FU. The disc was then dried at 50-60°C for 60 min in a scintillation vial mixed with 8 mL of Scintisole EX-H (Wako, Tokyo, Japan), and the radioactivity of produced [6-3H]-5FU was determined (13). OPRT activity was calculated from the amount of produced FUMP, the amount of which was proportional to radioactivity. Protein concentration in the enzyme solution was measured by the method of Lowry et al. (14).

Measurement of TdR-Pase expression. TdR-Pase expression was measured using an enzyme-linked immunosorbent assay (ELISA) (15). The tissue was homogenized and centrifuged at 105,000 g for 90 min. The supernatant was dialyzed overnight and then used as the source of crude TdR-Pase. Protein concentration was determined by the method of Lowry et al. (14). TdR-Pase activity was calibrated against those of standard solutions, and is presented as units per mg tissue protein. A 96-well microtiter plate (Nunc-immunoplate Maxisorp; Nunc, Roskilde, Denmark) was incubated overnight at 4°C with 10 μg/mL of the anti-DPD MoAb 4B9 in 10 mM phosphate-buffered saline (PBS, pH 7.6). The plate was then coated with 3% (w/v) skimmed milk in PBS (blocking buffer) for 1 h at room temperature, 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 (HCT 116 tumor homogenates serially diluted with blocking buffer) were dispensed onto the plate coated with antibody (16). The plate was subjected to four incubation steps: (1) at 37°C for 1 h followed by washing with 0.05% Tween 20 in PBS; (2) with mAb 232-2 at 1 μg/mL in blocking buffer for 1 h at 37°C and washing; (3) with 2000-fold diluted anti-mouse IgG conjugated with horseradish peroxidase (Bio-Rad, Hercules, Calif., USA) for 30 min at 37°C and washing; and finally (4) with a substrate solution containing 3,3’,5,5’-tetramethylbenzidine (TMB) and H₂O₂ (TMB Microwell Peroxidase Substrate System; KPL, Gaithersburg, MD, USA) for 10-20 min at room temperature. The peroxidase reaction was stopped by the addition of a 1M phosphate solution, and the amount of TdR-Pase sandwiched with the two anti-TdR-Pase MoAbs was estimated by measuring absorption at 450 nm with a plate reader (Bio-Rad, model 3550).

Measurement of DPD expression. DPD expression level was measured by a sandwich ELISA with two monoclonal antibodies (MoAbs) specific to human DPD as previously reported by Mori et al. (15). Briefly, a 96-well microtiter plate (Nunc-immunoplate Maxisorp, Nunc, Denmark) was incubated at 4°C overnight with 10 μg/mL of the anti-DPD MoAb 4B9 in 10 mM PBS (pH 7.6). The plate was coated with the antibody, incubated with 3% (w/v) skimmed milk in PBS (blocking buffer) for 2 h at 37°C, washed with dialyzed washing buffer (Kierkegaard & Perry Laboratories Inc., Gaithersburg, MD, USA) and kept at 4°C with 0.05% sodium azide PBS (ELISA plate). The ELISA plate was then washed three times, and then test samples and a serially diluted solution of the DPD standard, a homogenate of the HT-3 human cancer xenograft in PBS containing 0.3% (w/v) skimmed milk (DPD-diluent), were dispensed onto the ELISA. The plate was treated under the following four procedures: (a) incubation at 37°C for 1.5 h and then washing four times; (b) incubation with the anti-DPD MoAb 3A5 at 2 μg/mL in a blocking buffer for 1.5 h at room temperature and washing four times; (c) incubation with 1000-fold diluted rat antisem IgM (μ) conjugated with horseradish peroxidase (Zymed, So., San Francisco, CA, USA) for 1 h at room temperature and washing four times; and (d) incubation with a substrate solution containing 3,3’,5,5’-tetramethylbenzidine (TMB) and H₂O₂ (TMB Microwell Peroxidase Substrate System, Kierkegaard & Perry Laboratories Inc., Gaithersburg, MD, USA) for 10 min at room temperature. The peroxidase reaction was stopped by the addition of a 1 M phosphate solution, and the amount of DPD sandwiched with the two MoAbs was estimated by measuring its absorbance at 450 nm with a plate reader (Model 3550, Bio-Rad Laboratories).
The amount of DPD was calibrated with those obtained from the standard solution. DPD levels in tumor tissues were expressed in U/mg protein, in which one unit is equivalent to the amount of DPD protein that catabolizes 1 pmole of 5-FU per minute. The inter-assay precision of DPD ELISA was 2.5% in coefficient of variation.

In vitro chemosensitivity test, histoculture drug response assay (HDRA). HDRA was performed by a previously reported method (17, 18). Briefly, concentrations of 5-FU and CDHP in contact with cancer tissues were adjusted to 30 μg/mL (18) and 2.8 μg/mL in each well, these concentrations being 10 times the therapeutic peak plasma concentration achieved by intravenous administration at a clinical dose in each well (19). The antitumor effect of 5-FU was assessed using an in vitro HDRA technique in 99 cases. Cancer tissue was macroscopically identified and cut into pieces measuring approximately 2-3 mm in size. After weighing the individual pieces, they were placed on 1cm cubes of collagen sponge gel in the wells of a 24-well plate. Each tumor piece had been previously immersed in 800 μL complete medium and 200 μL of an anticancer drug solution, followed by incubation at 37˚C in a humidified atmosphere containing 5% CO2 for 7 days. Each drug was assessed in triplicate. The amount of DPD was calibrated with those obtained from the standard solution. DPD levels in tumor tissues were expressed in U/mg protein, in which one unit is equivalent to the amount of DPD protein that catabolizes 1 pmole of 5-FU per minute. The inter-assay precision of DPD ELISA was 2.5% in coefficient of variation.

Table II. 5-FU related enzyme activities in BUC specimens.

<table>
<thead>
<tr>
<th>Enzyme activity, median (interquartile range)</th>
<th>Normal tissue</th>
<th>Tumor tissue</th>
<th>P-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>OPRT (nmol/min/mg prot.)</td>
<td>0.117 (0.067-0.211)</td>
<td>0.176 (0.117-0.280)</td>
<td>p=0.0326</td>
</tr>
<tr>
<td>TdR-Pase (Unit/mg prot.)</td>
<td>30.6 (17.6-60.1)</td>
<td>54.0 (23.0-130.9)</td>
<td>p=0.0026</td>
</tr>
<tr>
<td>DPD (Unit/mg prot.)</td>
<td>49.8 (26.0-86.0)</td>
<td>74.2 (45.7-129.3)</td>
<td>p=0.0268</td>
</tr>
</tbody>
</table>

**Results**

**OPRT, TdR-Pase and DPD expression in BUC and normal bladder mucosa.** OPRT, TdR-Pase and DPD activities in UC tissue were significantly higher than those in normal bladder tissue (Table II).
Enhancement of sensitivity of BUC specimens to 5-FU using CDHP. Finally, the possibility that CDHP, a potential DPD inhibitor, might enhance the antitumor activity of 5-FU against BUC cells was also examined. A relatively small number of cases (n=17) showed a significant positive correlation between DPD activity and the increased rate of anticancer activity of 5-FU in combination with CDHP (Figure 4b), suggesting that the combination of 5-FU and a DPD inhibitor may contribute to overcoming 5-FU resistance in BUC cells, particularly in cases exhibiting higher DPD activity.

Discussion

In this study, it was found that OPRT activity was superior to that of TdR-Pase as a predictor of BUC sensitivity to 5-FU. Further, it was also observed that the calculated OPRT/DPD activity ratio was an even more useful predictor of 5-FU sensitivity than OPRT activity level alone, and that the combination of 5-FU and CDHP may be more effective than 5-FU alone in experimental settings. These findings may have implications with regards to the future use of 5-FU-related drugs for the treatment of BUC.

5-FU is metabolized by three phosphorylation pathways to the active metabolites which exert its antitumor effects (20, 21). Of these pathways, Fukushima et al. reported that the primary pathway involved orotate phosphoribosyl transferase (OPRT), and that 5-FU is initially phosphorylated through this pathway in about 80% of cancer cells regardless of histopathological characteristics (21). OPRT is thought to be the initial key enzyme in the 5-FU metabolic pathway and to play a major role in determining sensitivity to 5-FU in gastrointestinal malignancy (22, 23). In a previous preliminary study with a small number of cases, it was reported that OPRT activity may be a good indicator of the sensitivity of BUC to 5-FU (30), but there is little available data on the association between OPRT activity and the malignant potential or sensitivity of BUC to 5-FU.

With regards to the remaining two pathways, Fukushima et al. also reported that the second was catalyzed by uridine phosphorylase and uridine kinase and the third by thymidine phosphorylase (TdR-Pase) and thymidine kinase (21). TdR-Pase is identical to platelet-derived endothelial cell growth factor (PDECGF), which has potent angiogenic activity (24, 25), and a correlation between its activity and the malignant potential of UC has been shown (25, 26). However, Hirano et al. demonstrated that TdR-Pase activity was a significant predictor of the sensitivity of renal cell carcinoma (27) to 5-FU but not of UC (28).

Dihydropyrimidine dehydrogenase (DPD) catalyzes 5-FU to an inactive molecule, resulting in the loss of cytotoxicity (10), and an inverse correlation between DPD activity in BUC cells and their sensitivity to 5-FU has been reported (29). Mizutani et al. noted that DPD may be
associated with the malignant potential of BUC and play a role in 5-FU resistance through increased inactivation of 5-FU in cancer cells (29).

The presented findings that OPRT, TdR-Pase and DPD activity levels in BUC significantly differ from those in normal bladder specimens are consistent with previous reports (25, 29, 31). OPRT activity was not correlated with either histological grade or pathological stage of BUC, while TdR-Pase activity was significantly associated with both these histopathological factors. This finding likely explains why TdR-Pase is identical to PDECGF, and its potent angiogenic activity (24, 25). Aberrant angiogenesis occurs in several steps in tumor growth, including migration and proliferation of endothelial cells (24). In other words, higher TdR-Pase activity in tumor tissue is associated with higher malignant potential of the tumor itself, and several authors have in fact concluded that TdR-Pase activity is a good indicator of the malignant potential of UC (26) or renal cell carcinoma (32).

With regards to sensitivity to 5-FU, however, somewhat different findings were obtained, namely a significant positive correlation between OPRT activity level in BUC and sensitivity to 5-FU but no association for TdR-Pase. These results suggest that OPRT activity level is more important than TdR-Pase in predicting sensitivity to 5-FU, and appear consistent with findings that OPRT plays an important role in 5-FU sensitivity in various cancers (6, 7, 22, 23).

The present results are also consistent with the previous finding that OPRT activity might be a good indicator of the sensitivity of BUC to 5-FU, albeit that this analysis involved a relatively small population and did not include the other 5-FU-related enzymes (i.e. TdR-Pase and DPD) (30). In the present study, OPRT activity in T2-4 BUC cases with (n=2) and without recurrence (n=8) following administration of 5-FU-related compounds as postoperative adjuvant chemotherapy after curative surgery was also investigated. With a median postoperative observation period of 40.5 months (range 18 to 76 months) OPRT activity showed a tendency to be higher in the group without recurrence, albeit without statistical significance (data not shown), suggesting that patients with high OPRT activity may benefit more strongly from 5-FU than those with recurrence. Further investigation will be required to link in vitro results to the prediction of progression-free or long-term survival in patients with BUC receiving 5-FU-related compounds.

The present study investigated two methods which may identify an increased sensitivity of BUC specimens to 5-FU. The first is the calculated enzyme activity ratio, in particular the OPRT/DPD ratio. Given the impact on the efficacy of 5-FU, cancer cells with high OPRT and low DPD activity, namely a high OPRT/DPD ratio, might be theoretically more susceptible to 5-FU owing to the longer maintenance of higher active 5-FU concentrations. The results in fact showed

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**Figure 2. TdR-Pase activity among the three categories of histological grade and pathological stage.**

- **Grade**
  - **(Unit/mg prot.)**
    - Normal (n=39)
    - G1-2 (n=50)
    - G3 (n=52)
  - **p-values**
    - p=0.0043
    - p=0.627
    - p=0.043

- **Stage**
  - **(Unit/mg prot.)**
    - Normal (n=39)
    - Ta-1 (n=44)
    - T2-4 (n=58)
  - **p-values**
    - p=0.0017
    - p=0.915
    - p=0.0052
that the OPRT/DPD activity ratio had a stronger correlation coefficient with 5-FU sensitivity than OPRT activity alone. Ichikawa et al. showed that patients presenting a high OPRT/DPD ratio, based on the mRNA expression of each gene, survived for significantly longer than those with low ratio given tegafur-uracil, a 5-FU-related compound used for the treatment of metastatic colorectal cancer (7). Up till now, however, the association between OPRT/DPD ratio and sensitivity of BUC cells to 5-FU has not been reported. OPRT/DPD ratio appears to represent a useful indicator of the expected clinical efficacy of these compounds after curative surgery.
The second major point of the presented study was to examine whether CDHP possesses the potential to enhance 5-FU antitumor activity in BUC treatment. Despite the test being conducted in only 17 cases, analysis showed a significant correlation between DPD activity and an increasing rate of sensitivity to 5-FU. The superior cytotoxicity of S-1, a new oral 5-FU-related drug consisting of tegafur, CDHP and potassium oxonate in a molar ratio of 1:0.4:1 (8), appears to be attributable to both the increased inhibition of DNA synthesis and enhanced blockade of RNA function against tumors with high DPD activity (9). These findings were derived from experiments using 4 human gastric cancer xenografts, but may nevertheless suggest the presence of good responders to S-1 among BUC patients. Further studies to verify clinical outcomes are warranted.

In conclusion, the presented study demonstrates that OPRT is the most important enzyme involved in the three 5-FU phosphorylation pathways. Further, this enzyme is useful in predicting the sensitivity of BUC to 5-FU, but not the malignant potential of BUC. Accordingly, assessment of OPRT activity may be useful in the clinical management of BUC, especially for patients who receive 5-FU-related compounds after curative surgery. Moreover, OPRT/DPD activity ratio is probably more useful in predicting 5-FU efficacy than OPRT activity alone. It was also found that CDHP is conducive to improving the sensitivity of BUC to 5-FU, especially in cases with higher DPD activity. Although further investigation to verify the association between these in vitro data and subsequent clinical outcomes is required, this study may have implications for tailor-made medication using 5-FU-related compounds for BUC.

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1008