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The significance of dihydropyrimidine dehydrogenase (DPD) activity in bladder cancer

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Abstract

Dihydropyrimidine dehydrogenase (DPD) is the rate-limiting enzyme in the pathway of uracil and thymine catabolism. DPD is also the principal enzyme involved in the degradation of 5-fluorouracil (5-FU), which is one of the anticancer chemotherapeutic agents currently used in the treatment of bladder cancer. Little is known about the significance of DPD activity in human cancers. We investigated the activity of DPD in 74 bladder cancers and the relationship between the DPD activity and the sensitivity to 5-FU. The levels of DPD activity in bladder cancer and normal bladder tissues were determined by the 5-FU degradation assay. The sensitivity to 5-FU was assessed by the microculture tetrazolium dye (dimethylthiazolyl-2-5-diphenyltetrazolium bromide; MTT) assay. The activity of DPD was approximately 2-fold higher in bladder cancer tissues compared with normal bladder tissues. DPD activity in invasive bladder cancers was approximately 2-fold higher than that in superficial cancers. In addition, the levels of DPD activity in grade 2 and grade 3 bladder cancers were approximately 3-fold and 4-fold higher than that in grade 1 cancers, respectively. Patients with superficial bladder cancer with a low DPD activity had a slightly longer postoperative tumour-free period than those with a high DPD activity over a 2-year follow-up period, but this was not significant. There was an inverse correlation between DPD activity in bladder cancer cells and their sensitivity to 5-FU. Furthermore, 5-chloro-2,4-dihydroxypyridine (CDHP), a potent DPD inhibitor, enhanced the sensitivity to 5-FU. The present study has demonstrated that the level of DPD activity correlated with the progression of the stage and an increase in the grade of the bladder cancer. These results suggest that an elevated DPD activity might be associated with the malignant potential of the bladder cancer. In addition, it might be possible to overcome 5-FU insensitivity by using DPD inhibitors in the treatment protocols of 5-FU-based chemotherapy for bladder cancers.

Keywords: Bladder cancer; DPD; 5-FU

1. Introduction

5-fluorouracil (5-FU) is widely used in the treatment of various cancers including bladder cancer [1,2]. 5-FU itself is inactive and requires intracellular conversion to form cytotoxic nucleotides. The nucleotides exert their cytotoxic effects through inhibition of thymidylate synthase (TS) activity and incorporation into RNA and/or DNA. However, the role of catabolism of 5-FU has not been appreciated until recently. Most administered 5-FU is degraded through a catabolic pathway with dihydropyrimidine dehydrogenase (DPD) [3,4]. The efficacy of 5-FU is related to the plasma level of this agent, which is inversely related to the level of DPD activity [5,6]. Therefore, catabolism of 5-FU may represent a major determinant of the anticancer activity of 5-FU. Indeed, several DPD inhibitors are under evaluation as modulators of 5-FU treatment [7,8].

Human DPD was purified from the liver and the cDNA was cloned and sequenced [9,10]. The DPYD gene is localised to the centrometric region of human chromosome 1 between 1p22 and q21. DPD is the initial and rate-limiting enzyme in the three-step pathway of uracil and thymine catabolism, leading to the formation of β-alanine. The enzyme activity is high in the liver and peripheral blood mononuclear cells and was very vari-

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able in cancers [11]. DPD activity has been measured in surgical specimens from head and neck cancers and colorectal cancers [12,13]. The activities ranged from 13 to 193 pmol/min/mg protein and from 28 to 207 pmol/min/mg protein, respectively. However, the data on DPD activity in cancers are limited and little is known about the significance of DPD activity in cancer biology. In this study, we measured the activity of DPD in primary bladder cancers and evaluated the relationship between DPD activity and the grade or stage of the bladder cancer. In addition, we investigated whether the activity of DPD in bladder cancer cells was associated with their sensitivity to 5-FU.

2. Patients and methods

2.1. Patients

Surgical specimens were obtained from 74 patients with primary bladder cancer (at different times of the day). They included 51 male (69%) and 23 female patients (31%), ranging in age from 23 to 86 years. Histological diagnosis revealed that all patients had transitional cell carcinoma. Their histological classification and staging according to the TNM classification were: Grade $(G)_1$ ($n=15$; 20%), G_2 ($n=31$; 42%), G_3 ($n=28$; 38%), and T_a ($n=36$; 49%), T_1 ($n=17$; 23%), T_2 ($n=8$; 11%), T_3 ($n=9$; 12%), T_4 ($n=4$; 5%), respectively. Samples of normal bladder were collected from 18 bladder cancer patients undergoing radical cystectomy. The normal bladder tissues were separated from normal urothelium as much as possible and were checked pathologically. The specimens were stored frozen at −80°C until use for the assay of DPD activity.

2.2. Reagents

[6-^{3}H] 5-FU was obtained from the Japan Radioisotope Association, Tokyo, Japan. 5-FU (Lot No. 308033) was kindly supplied by Kyowa Hakkou Co. Ltd, Tokyo, Japan. 5-chloro-2,4-dihydroxypyridine (CDHP) was a gift from Taiho Pharmaceutical Co. Ltd, Tokyo, Japan.

2.3. Tumour cells

The T24, J82 and HT1197 human bladder cancer cell lines were maintained in monolayers on plastic dishes in Roswell Park Memorial Institute (RPMI)-1640 medium (Gibco, Bio-cult, Glasgow, UK) supplemented with 25 mM HEPES (Gibco), 2 mM L-glutamine (Gibco), 1% non-essential amino acid (Gibco), 100 units/ml penicillin (Gibco), 100 μg/ml streptomycin (Gibco) and 10% heat-inactivated fetal bovine serum (FBS: Gibco), hereafter referred to as complete medium [14].

Fresh bladder cancer cells derived from 17 patients were separated from surgical specimens for *in vitro* primary culture as previously described [15,16]. Briefly, cell suspensions were prepared by treating finely minced cancer tissues with collagenase (3 mg/ml, Sigma Chemical Co., St Louis, USA). After washing in RPMI-1640 medium, the cell suspensions were layered on discontinuous gradients consisting of 2 ml of 100%, 2 ml of 80% and 2 ml of 50% Ficoll-Hypaque in 15-ml plastic tubes and were centrifuged at 400*g* for 30 min. Lymphocyte-rich mononuclear cells were collected from the 100% interface, cancer cells and mesothelial cells from the 80% interface. Cell suspensions enriched with cancer cells were sometimes 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, 15 and 10% Percoll in complete medium in 15-ml plastic tubes and centrifuged them for 7 min at 25*g* at room temperature. Cancer cells depleted of lymphoid cells were collected from the bottom, washed, and suspended in complete medium. Cancer cells were more than 93% viable on average according to the trypan blue dye-exclusion test. The cancer cells were maintained in monolayers on plastic dishes in complete medium.

2.4. Measurement of DPD activity in bladder cancer and normal bladder tissues

Bladder cancer and normal bladder tissues were homogenised in 4 volumes of 50 mM Tris–HCl (pH 8.0) containing 5 mM 2-mercaptoethanol, 25 mM KCl and 5 mM $MgCl_2$. The homogenate was centrifuged at 105 000*g* for 1 h at 4°C, and the supernatant was used for the measurement of DPD activity as described before in Ref. [17]. Briefly, the assay mixture, in a final volume of 0.25 ml, consisted of 50 mM Tris–HCl (pH 8.0), 10 mM $MgCl_2$, 25 mM NaF, 50 mM nicotinamide, 5 mM adencine triphosphate (ATP), 1 mM NADPH, [6-^{3}H] 5-FU (0.2 μCi, 20 μM) and the enzyme extract (0.1 ml). The mixture was incubated for 30 min at 37°C and the reaction was stopped by heating at 100°C in a water-bath. After centrifugation at 800*g*, the supernatant (0.1 ml) was treated with 0.01 ml of 2 M KOH for 30 min at room temperature. Then, the mixture was treated with 0.005 ml of 2 M PCA and centrifuged. An aliquot (20 μl) of the supernatant was spotted onto a thin layer chromatography plate (Merck silica gel $60F_{254}$ precoated plate, 2.5×10 cm, thickness 0.25 mm) and developed with a mixture of chloroform, methanol and acetic acid (17:3:1, v/v/v). The spots of 2-fluoro-β-alanine and 2-fluoro-β-ureidopropionic acid, 5-FU degradation products, were scraped into vials and mixed with 10 ml of ACS-II scintillation fluid (Amersham). The radioactivity was measured in a Wallac 1410 liquid scintillation counter (Pharmacia). This method makes it

possible to estimate DPD activity higher than 0.4 pmol/mg protein/min. The standard deviations (S.D.) were less than 8% of the mean values, when the level of DPD activity was more than 0.4 pmol/mg protein/min. Repeated measurements yielded the same results.

This method is simpler and more convenient than the high performance liquid chromatography (HPLC) method described by Diasio and colleagues [18].

DPD activity greater than the median value (2.43 pmol/mg protein/min) was regarded as high activity and DPD activity less than the median value was regarded as low activity.

2.5. Cytotoxicity assay

The microculture tetrazolium dye (dimethylthiazolyl-2,5-diphenyltetrazolium bromide; MTT) assay was used to determine tumour cell lysis as previously described in Ref. [19]. Briefly, 100 μl of the target cell suspension (2×10^4 cells) was added to a well of a 96-well flat-bottom microtitre plate (Corning Glass Works, Corning, NY, USA), and each plate was incubated for 24 h at 37°C in a humidified 5% CO_2 atmosphere. After incubation, the supernatant was aspirated and tumour cells were washed three times with RPMI medium, and 200 μl of drug solution or complete medium for controls were distributed in the 96-well plates. Each plate was incubated for 24 h at 37°C. Following incubation, 20 μl of MTT working solution (5 mg/ml, Sigma Chemical Co.) was added to each culture well and the cultures were incubated for 4 h 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 Co.) supplemented with 0.05 N HCl. The absorbance of each well was measured with a microculture plate reader (Immunoreader, Japan Intermed Co. Ltd, Tokyo, Japan) at 540 nm. The per cent cytotoxicity was calculated by the following formula: Percentage cytotoxicity = [1−(absorbance of experimental wells/absorbance of control wells)]×100.

2.6. Statistical analysis

All determinations were made in triplicate. For statistical analysis, Student's *t*-test and Pearson's correlation test were used. Postoperative disease-free interval was determined by the Kaplan–Meier method. The generalised Wilcoxon test and the Cox–Mantel test were used to establish the statistical difference in recurrence between the patients with high and low levels of DPD activity. A *P* value of 0.05 or less was considered significant.

3. Results

3.1. DPD activity in bladder cancer and normal bladder tissues

Fig. 1 shows the DPD activity in bladder cancer and normal bladder tissues. The DPD activity in the bladder cancer tissues was approximately 2-fold higher than that in the normal bladder tissues.

3.2. The level of DPD activity in the bladder cancers

The activity of DPD was approximately 2-fold higher in the invasive bladder cancers (T_{2-4}) than that in superficial cancers (T_a, T_1) (Fig. 2). DPD activity in the Grade 2 bladder cancers was approximately 3-fold higher than that in the Grade 1 cancers (Fig. 3). Furthermore, DPD activity in the Grade 3 bladder cancers

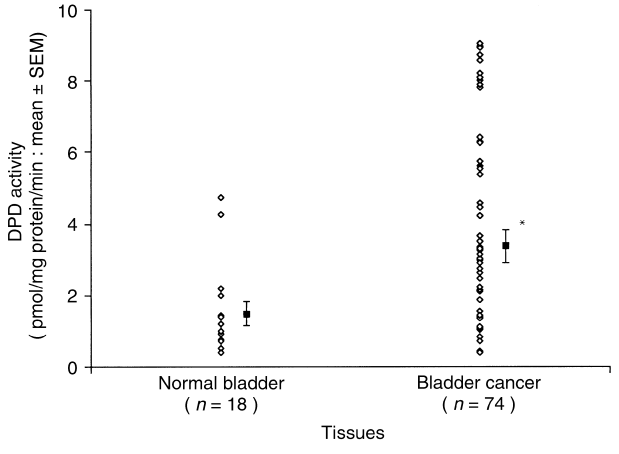

Fig. 1. The levels of dihydropropyrimidine dehydrogenase (DPD) activity in bladder cancer and normal bladder tissues. The levels of DPD activity in bladder cancer and normal bladder tissues were quantitated by the 5-fluorouracil (5-FU) degradation assay. SEM, standard error of the mean. *$P<0.05$ versus normal bladder.

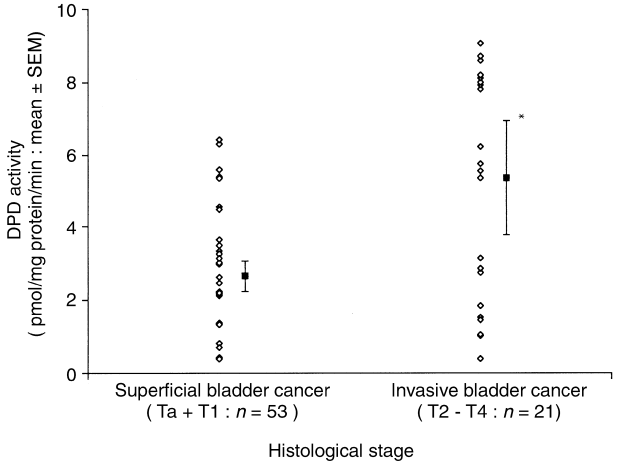

Fig. 2. The levels of dihydropropyrimidine dehydrogenase (DPD) activity according to the histological stage of the bladder cancer. The levels of DPD activity in bladder cancer were quantitated by the 5-fluorouracil (5-FU) degradation assay. SEM, standard error of the mean. *$P<0.05$ versus superficial bladder cancer.

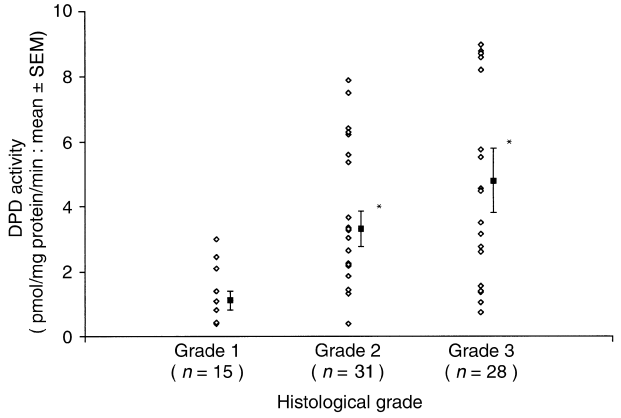

Fig. 3. The levels of dihydropropyrimidine dehydrogenase (DPD) activity according to the histological grade of the bladder cancer. The levels of DPD activity in bladder cancer were quantitated by the 5-fluorouracil (5-FU) degradation assay. SEM, standard error of the mean. *$P < 0.05$ versus Grade 1.

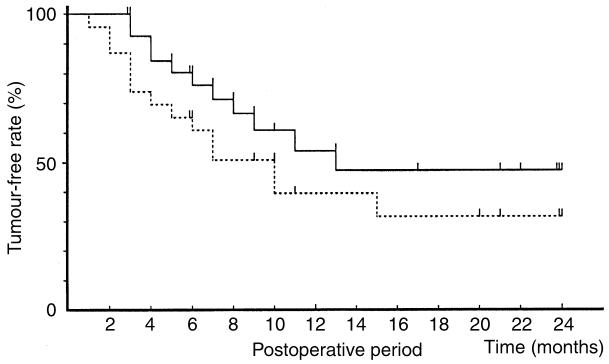

Fig. 4. Postoperative tumour-free period of patients with superficial bladder cancer. Postoperative tumour-free period of superficial bladder cancer patients undergoing transurethral resection was determined by the Kaplan–Meier method. Dihydropropyrimidine dehydrogenase (DPD) activity greater than the median value was regarded as high activity and activity less than the median value was regarded as low activity. —, 27 patients with low DPD activity; - - - -, 26 patients with high DPD activity.

was approximately 4-fold higher than that in the Grade 1 cancers. Thus, DPD activity increased with the invasiveness and decreasing differentiation of the tumours as represented by the stage and grade of the bladder cancers, respectively.

3.3. The relationship between DPD activity and the postoperative tumour-free period in patients with superficial bladder cancer

Superficial bladder cancer patients undergoing transurethral resection were evaluated for the postoperative clinical course. These patients received surgery alone without adjuvant chemotherapy. The patients included 36 patients with T_a bladder cancer and 17 patients with T_1 cancer. The postoperative tumour-free period was estimated by Kaplan–Meier analysis. Patients with bladder cancer were divided into two groups, namely, those with high DPD activity (greater than the median value (2.43 pmol/mg protein/min)) and those with low activity (less than the median value). The mean follow-up period of the patients was 25 months. Patients with low DPD activity had a slightly longer tumour-free interval compared with those with high DPD activity during the 2-year follow-up, but this was not significant (Fig. 4). These results suggest that DPD activity might be a prognostic indicator in patients with superficial bladder cancer and that a low DPD activity might be associated with a good prognosis.

3.4. Correlation between the DPD activity in bladder cancer cells and their sensitivity to 5-FU

DPD is the principal enzyme involved in the degradation of 5-FU [3,4]. The activity of DPD is highly correlated with 5-FU pharmacokinetics [5,6]. We examined the association between the DPD activity in bladder cancer cells and their sensitivity to 5-FU. Fourteen primary cultures derived from surgical specimens and three bladder cancer lines were studied. Fig. 5 demonstrates that there was an inverse correlation between the DPD activity in the bladder cancer cells and their sensitivity to 5-FU. Similar findings were observed with different doses of 5-FU (data not shown).

3.5. Overcoming the 5-FU resistance of bladder cancer cells using CDHP

Various inhibitors of DPD activity have been developed to increase the anticancer effect of 5-FU [7,8]. CDHP is a potent DPD inhibitor and does not show anticancer activity by itself [17]. We examined whether

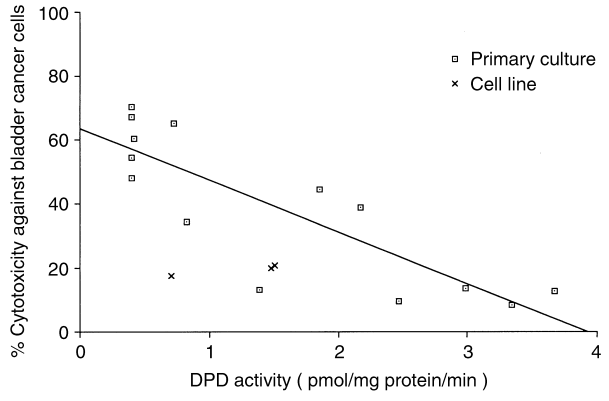

Fig. 5. Relationship between dihydropropyrimidine dehydrogenase (DPD) activity in bladder cancer cells and their sensitivity to 5-fluorouracil (5-FU). Bladder cancer cells of 14 primary cultures and three cell lines were used as target cells. A significant correlation between the level of DPD activity of bladder cancer cells and their sensitivity to 5-FU (10 μM) was observed ($n = 17$, $r = 0.735$, $P < 0.001$ by Pearson's correlation test).

Table 1
Enhancement of the sensitivity of bladder cancer cells to 5-FU by CDHP

Drugs	% Cytotoxicity (mean±S.D.)[a]
5-FU	38.2±16.9
5-FU + CDHP	66.8±14.7[b]

$n = 17$. S.D., standard deviation.

[a] The direct cytotoxic effect of 5-fluorouracil (5-FU) (10 μM) and/or 5-chloro-2,4-dihydroxypyridine (CDHP) (10 μM) on freshly separated bladder cancer cells was assessed by an 1-day microculture tetrazolium dye (dimethylthiazolyl-2,5-diphenyl tetrazolium bromide; MTT) assay. Bladder cancer cells were derived from 17 patients with bladder cancer.

[b] $P < 0.05$ versus 5-FU alone.

CDHP enhanced the cytotoxicity of 5-FU against bladder cancer cells. CDHP significantly augmented the cytotoxic activity of 5-FU (Table 1). Similar findings were observed with different doses of CDHP and 5-FU (data not shown). These results suggest that the use of DPD inhibitors may be one therapeutic option to overcome the 5-FU resistance of bladder cancer cells.

4. Discussion

In the present study, we demonstrated that DPD activity was upregulated in bladder cancer tissues, compared with normal bladder tissues and that the level of DPD activity correlated with progression of the stage and an increase in the grade of bladder cancer. Although we reported only a small number of patients, our data suggest that DPD activity might play an important role in mediating the malignant potential of bladder cancer.

DPD activity in the bladder cancer tissues was approximately 2-fold higher than that in the normal bladder tissues. DPD activity in the cancer tissues was up to 6.6-fold greater than that in adjacent normal tissue in patients with head and neck cancer and 3.3-fold greater in patients with colorectal cancer [12,13]. This high DPD activity in the cancer tissues compared with normal tissues may contribute to the unfavourable differential between the anticancer effect and the adverse effect of 5-FU. Thus, a higher degree of 5-FU degradation may occur in cancer tissues, compared with that in normal tissues. These findings suggest that DPD may play a role in 5-FU resistance through the increased inactivation of 5-FU in cancer cells.

It has been reported that there is a circadian variability in the DPD activity measured in blood lymphocytes from humans, with a 2-fold ratio between maximum and minimum activity [5]. For reasons of feasibility, no data are available on DPD circadian variability in other human tissues. Thus, a possible circadian variability in tumoral and non-tumoral tissues can not be ruled out. In the present study, tumoral and non-tumoral specimens of bladder cancer patients undergoing radical cystectomy were sampled at the same time, but at different times of the day for each patient. Nevertheless, the mean level of DPD activity in the tumoral lesions was higher than that in the normal lesions. These findings suggest that circadian variation of DPD activity in bladder tumoral and non-tumoral tissues might not be critical.

The high expression of other drug-metabolising enzymes in bladder cancer have been described. The expression of glutathione and its related enzymes, which are associated with the resistance to anticancer cytotoxic agents, are observed, often at high levels, in bladder cancer cells [20,21]. Metallothionein is known to bind cisplatin, as well as various metals, and this binding is thought to protect cells against the toxic effects of the cytotoxic drugs [22]. Bladder cancer cells also express high levels of metallothionein [23].

The present study demonstrated that DPD activity in bladder cancer cells inversely correlated with their sensitivity to 5-FU and that CDHP enhanced the sensitivity to 5-FU. Thus, these data provide a pharmacological rationale for the potential use of DPD inhibitors as 5-FU modulators. Interferon-α inhibits DPD activity and has been used in combination with 5-FU [24,25]. 5-Benzyloxybenzyluracil, a strong DPD inhibitor, increased 5-FU cytotoxicity in human colon tumour xenografts [8]. Another promising DPD inhibitor, 5-ethynyluracil improved the efficacy and therapeutic index of 5-FU [7]. Altogether, these findings suggest that 5-FU catabolism in bladder cancer cells may be one of the factors determining 5-FU responsiveness and justify the clinical use of specific DPD inhibitors, although resistance to 5-FU is likely to be multifactorial.

Recently, we have developed an oral 5-FU derivative (S-1) for clinical use, which consists of tegafur, CDHP and potassium oxonate in a molar ratio of 1:0.4:1 [17,26]. CDHP does not have antitumour activity by itself and plays a role as a biochemical modulator, which competitively inhibits DPD activity. Tegafur is a prodrug of 5-FU. S-1 had a better therapeutic effect on various rat cancers and human xenografts than other orally administered (p.o.) fluoropyrimidines. The current findings suggest that S-1 might be a potential anticancer agent against bladder cancer.

Many experimental studies performed on various cancers have demonstrated that either overexpression of TS protein or TS activity is associated with 5-FU resistance [27,28]. These studies have shown an inverse correlation between TS expression and the response rate obtained to 5-FU-containing chemotherapy. Since TS is the main target enzyme for 5-FU, the measurement of TS expression, as well as DPD activity may be necessary

for the evaluation of the efficacy of 5-FU-based chemotherapy.

We used thin layer chromatography (TLC) to measure DPD activity. The DPD enzyme activity detected by the TLC assay correlated with *DPYD* gene expression in semiquantitative PCR analysis [29]. The DPD activity is also associated with the level of DPD as determined by western blot analysis [30]. In addition, preliminary experiments demonstrated that the DPD activity is related to DPD expression measured by enzyme-linked immunosorbent assay (ELISA). These findings suggest that DPD activity might be correlated with *DPYD* mRNA and protein levels.

In conclusion, the current study has demonstrated that the DPD activity level in bladder cancer paralleled the increase in histological stage and grade, and that an elevated level of DPD activity might be associated with an early recurrence in patients with superficial bladder cancer. These findings suggest that the assessment of DPD activity might be useful both in the management and the treatment of bladder cancer. Since the level of DPD activity might be associated with the malignant potential of bladder cancer, the use of DPD activity levels might help to select patients with a potentially poor prognosis for more intensive surgical or chemotherapeutic approaches. Furthermore, the combination of 5-FU and DPD inhibitors might be effective for the treatment of bladder cancer. However, further analysis is needed on the regulatory effects of DPD activity and the *in vivo* combined effect of 5-FU and DPD inhibitors. It would also be interesting to know if DPD activity varies according to the cell cycle phase and studies are currently underway.

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