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European Journal of Cancer 39 (2003) 541–547

European Journal of Cancer

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Significance of dihydropyrimidine dehydrogenase activity in renal cell carcinoma

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Received 15 March 2002; received in revised form 27 August 2002; accepted 30 August 2002

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), an anticancer chemotherapeutic agent that is used clinically to treat renal cell carcinoma (RCC). Little is known about the significance of DPD activity in human cancers. We investigated the activity of DPD in 68 RCC and the relationship between DPD activity and the sensitivity to 5-FU. The levels of DPD activity in RCC and normal kidney samples were determined by the 5-FU degradation assay. The sensitivity to 5-FU was assessed by the microculture tetrazolium dye assay. The activity of DPD was approximately 2-fold higher in normal kidney compared with RCC. DPD activity in Stage I/II RCC was approximately 2-fold higher than that in Stage III/IV RCC. The levels of DPD activity in Grade 1 and Grade 2 RCC were 3 and 2-fold higher, respectively, than that in the Grade 3 cancers. There was an inverse correlation between DPD activity in RCC 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 current study is the first to demonstrate that the level of DPD activity was inversely correlated with both the progression of the disease and increased grade of RCC, and that DPD activity was inversely associated with the sensitivity of RCC to 5-FU, which was enhanced by a DPD inhibitor. These results suggest that a low DPD activity may be associated with the malignant potential of RCC. In addition, it may be possible to overcome 5-FU resistance by using DPD inhibitors in the treatment protocols of 5-FU-based chemotherapy for RCC.

Keywords: Renal cell carcinoma; DPD; 5-FU; CDHP

1. Introduction

5-Fluorouracil (5-FU) is commonly used in the treatment of various cancers including renal cell carcinoma (RCC) [1,2]. 5-FU itself is inactive and requires intracellular conversion to form cytotoxic nucleotides. The nucleotides exert their cytotoxic effects through the inhibition of thymidylate synthase (TS) activity and incorporation into RNA and DNA. However, the role of catabolism of 5-FU has not been elucidated until recently. Most of the administered 5-FU is degraded through the catabolic pathway with dihydropyrimidine dehydrogenase (DPD) [3,4]. The efficacy of 5-FU is related to plasma level of this agent, which is inversely associated with the level of DPD activity [5,6]. Therefore, the catabolism of 5-FU may be a major determinant of its anticancer activity. 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

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0959-8049/03/$ - see front matter
PII: S0959-8049(02)00730-X

peripheral blood mononuclear cells and has been shown to be very variable in cancers [11]. DPD activity has been measured in surgical specimens from head and neck cancers and colorectal cancers [12,13]. Activities ranged from 13 to 193 pmol/mg protein/min and from 28 to 207 pmol/mg protein/min, 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 the present study, we examined DPD activity in RCC and normal kidney tissues and evaluated the relationship between the level of DPD activity and the stage/grade status of RCC. In addition, we investigated whether DPD activity in RCC was related to the sensitivity to 5-FU.

2. Materials and methods

2.1. Patients

Surgical specimens were obtained from 68 patients with RCC. Informed consent was obtained from each patient. They included 51 male and 17 female patients, ranging in age from 32 to 82 years. Histological diagnosis revealed that 63 and 5 patients had clear cell carcinoma and papillary RCC, respectively. Their histological classification and staging according to the TNM classification (fifth edition, 1997) were: T1 ($n=47$), T2 ($n=11$), T3 ($n=9$), T4 ($n=1$); N0 ($n=64$), N1 ($n=2$), N2 ($n=2$); M0 ($n=62$), M1 ($n=6$); Stage I ($n=47$), Stage II ($n=9$), Stage III ($n=5$), Stage IV ($n=7$), and G1 ($n=11$), G2 ($n=51$), G3 ($n=6$), respectively. Samples of normal kidneys were also collected from the same patients. The specimens were stored frozen at −80 °C until use for the measurement 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 the Taiho Pharmaceutical Co. Ltd., Tokyo, Japan.

2.3. Tumour cells

The NC65 and Caki-1 RCC cell lines were maintained in monolayers on plastic dishes in Roswell Park Memorial Institute (RPMI)-1640 medium (Gibco, Bio-cult, Glasgow, Scotland, UK) supplemented with 25 mM HEPES (Gibco), 2 mM L-glutamine (Gibco), 1% non-essential amino acids (Gibco), 100 units/ml penicillin (Gibco), 100 μg/ml streptomycin (Gibco) and 10% heat-inactivated fetal bovine serum (Gibco), hereafter referred to as complete medium [14,15].

Fresh RCC cells derived from 23 patients were separated from surgical specimens for in vitro primary culture as previously described in Refs. [16,17]. Their histological staging according to the TNM classification (fifth edition, 1997) were Stage I ($n=17$), Stage II ($n=2$), Stage III ($n=1$), Stage IV ($n=3$). The mean level of DPD activity was 10.1 pmol/mg protein/min (range: 1.5–53.2 pmol/mg protein/min). 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% (v/v), 2 ml of 80% (v/v) and 2 ml of 50% (v/v) Ficoll-Hypaque in 15-ml plastic tubes and were centrifuged at 400g 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 (v/v), 15 (v/v), and 10% (v/v) Percoll in complete medium in 15-ml plastic tubes and centrifuged them for 7 min at 25g at room temperature. Cancer cells depleted of lymphoid cells were collected from the bottom, washed, and suspended in complete medium. Cancer cells were (on average) more than 93% viable according to the trypan blue dye-exclusion test. The cancer cells were maintained in monolayers on plastic dishes in complete medium and grew well. The cancer cells of the primary cultures were used as target cells for lysis of 5-FU and CDHP in the microculture tetrazolium dye (MTT) assay.

2.4. Measurement of DPD activity in RCC and normal kidney samples

RCC and normal kidney samples 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 000g for 1 h at 4 °C, and the supernatant fluid was used for the measurement of DPD activity as described before in Ref. [18]. Briefly, the assay mixture, in a final volume of 250 μl, consisted of 50 mM Tris–HCl (pH 8.0), 10 mM $MgCl_2$, 25 mM NaF, 50 mM nicotinamide, 5 mM ATP, 1 mM NADPH, [6-^{3}H] 5-FU (0.2 μCi, 20 μM) and the enzyme extract (100 μl). 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 3000 rpm, the supernatant (100 μl) was treated with 10 μl of 2 M KOH for 30 min at room temperature. Then, the mixture was treated with 5 μl of 2 M PCA and centrifuged. An aliquot (20 μl) of the supernatant was spotted onto a thin layer chromatography plate (TLC

plate: Merck silica gel $60F_{254}$ precoated plate, 2.5×10 cm, thickness 0.25 mm: Merk, Whitehouse Station, NJ, USA) 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 according to the molecular weight and mixed with 10 ml of ACS-II scintillation fluid (Amersham, Buckinghamshire, UK). The radioactivity was measured in a Wallac 1410 liquid scintillation counter (Pharmacia, Uppsala, Sweden). Internal standards were used to compare assays. This method made it possible to estimate DPD activity higher than 0.4 pmol/mg protein/min. Repeated measurements yielded the same results.

2.5. Cytotoxicity assay

The MTT assay was used to determine tumour cell lysis as previously described in Refs. [19,20]. Briefly, 100 μl of target cell suspension (2×10^4 cells) were added to each well of 96-well flat-bottomed microtitre plates (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 percent cytotoxicity was calculated using 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 analyses, the Student's *t*-test and Pearson's correlation test were used. A *P* value of 0.05 or less was considered significant.

3. Results

3.1. DPD activity in RCC and normal kidney

Fig. 1 shows the values of DPD activity in specimens from patients with RCC. The DPD activity in normal kidney samples was approximately 2-fold higher than that in the RCC samples and this finding was significant. The level of DPD activity in the normal kidney samples in patients with RCC was also similar to that in patients with renal pelvic cancer or ureteral cancer (data not shown).

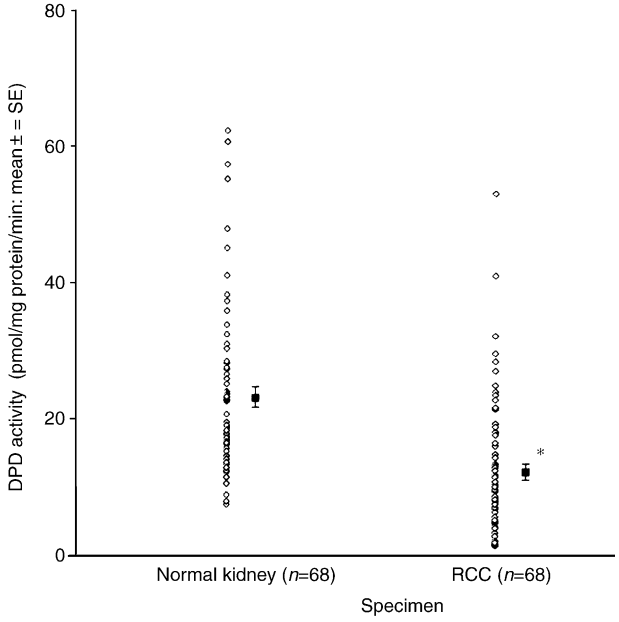

Fig. 1. The levels of DPD activity in RCC and normal kidney specimens. Dihydropyrimidine dehydrogenase (DPD) activity in renal cell carcinoma (RCC) and normal kidney samples was quantitated by the 5-fluorouracil (5-FU) degradation assay as described in Materials and methods. * $P < 0.05$ versus normal kidney. S.E., standard error.

3.2. The level of DPD activity in RCC

The activity of DPD was approximately 2-fold higher in the T1 RCC samples than that in T3 RCC samples (Table 1). Furthermore, DPD activity in T1/2 RCC samples was also approximately 2-fold higher than that in T3/4 RCC samples. The level of DPD activity in M0 RCC samples was approximately 3-fold higher than that in M1 RCC samples (Table 1). The activity of DPD was 2.5-fold higher in Stage I RCC than that in Stage IV RCC samples (Fig. 2). In addition, DPD activity in Stage I/II RCC was 2-fold higher than that in Stage III/IV RCC samples.

The levels of DPD activity in Grade 1 and Grade 2 RCC samples were 3-fold and 2-fold higher respectively, than that in the Grade 3 cancers (Fig. 3). DPD activity in papillary RCC samples was 3-fold higher than that in the clear cell RCC samples (Fig. 4).

Thus, DPD activity was reduced as the disease progressed and the histological grade increased. These results suggest that high DPD activity may be marker of good prognosis.

Table 1
The levels of DPD activity according to TNM classification of RCC

TNM classification	DPD activity (pmol/mg protein/min: mean±S.E.)[a] (range)
T1 ($n=47$)	14.4±1.5 (1.7–53.2)
T2 ($n=11$)	10.6±2.6 (1.8–29.6)
T3 ($n=9$)	6.8±2.3[b] (1.5–19.4)
T4 ($n=1$)	13.1
T1/2 ($n=58$)	13.7±1.3 (1.7–53.2)
T3/4 ($n=10$)	7.4±2.1[c] (1.5–29.6)
M0 ($n=62$)	13.6±1.3 (1.5–53.2)
M1 ($n=6$)	4.5±1.3[d] (1.8–9.4)

[a] The levels of DPD activity in RCC were quantitated by the 5-FU degradation assay as described in Materials and methods.
[b] $P<0.05$ versus T1.
[c] $P<0.05$ versus T1/2.
[d] $P<0.05$ versus M0.

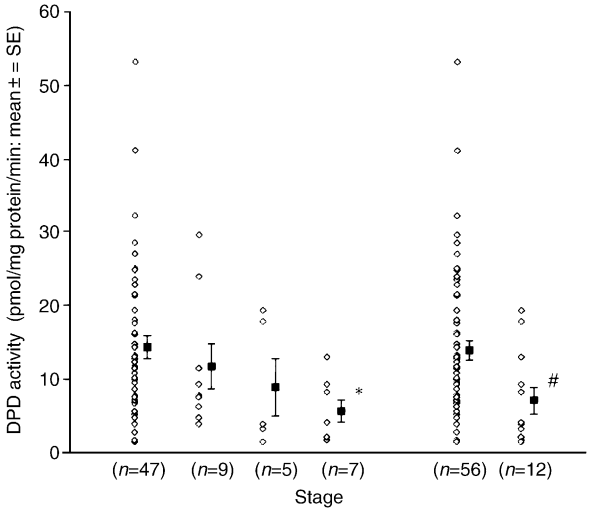

Fig. 2. The levels of DPD activity according to Stage of RCC. The levels of DPD activity in the RCC samples were quantitated by the 5-FU degradation assay as described in Materials and methods. * $P<0.05$ versus Stage I, # $P<0.05$ versus Stage I/II.

3.3. Correlation between the level of DPD activity in RCC cells and their sensitivity to 5-FU

DPD is the principal enzyme involved in the degradation of 5-FU [3,4]. Several reports suggest that the activity of DPD may correlate with 5-FU pharmacokinetics [5,6,21,22]. We then examined the association between the level of DPD activity in the RCC cells and their sensitivity to 5-FU. Twenty-three primary cultures derived from surgical specimens and two RCC cell lines

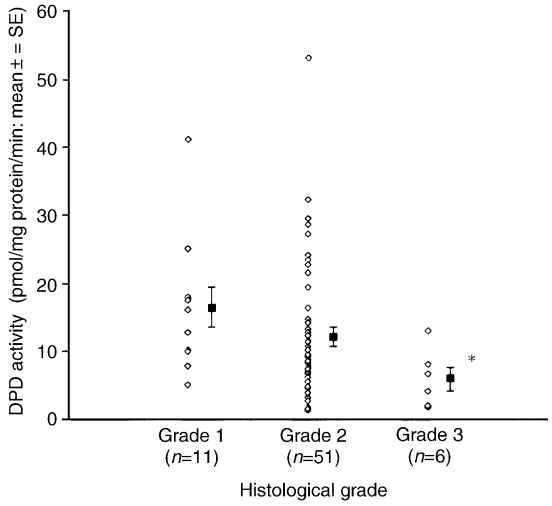

Fig. 3. The levels of DPD activity according to histological grade of RCC. The levels of DPD activity in the RCC samples were quantitated by the 5-FU degradation assay as described in Materials and methods. * $P<0.05$ versus Grade 1, Grade 2.

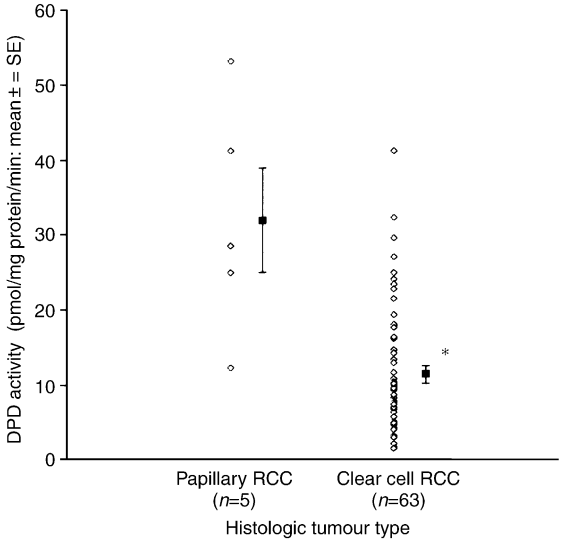

Fig. 4. The levels of DPD activity according to histological tumour type of RCC. The levels of DPD activity in RCC were quantitated by the 5-FU degradation assay as described in Materials and methods. * $P<0.05$ versus papillary RCC.

were used. Fig. 5 demonstrates that there was an inverse correlation between the level of DPD activity in the RCC cells and their sensitivity to 5-FU. Similar findings were observed with different doses of 5-FU (data not shown).

3.4. Overcoming the resistance of RCC cells to 5-FU using CDHP

Various inhibitors of DPD activity have been developed to increase the anticancer effects of 5-FU [7,8]. CDHP is one of the most potent DPD inhibitors and does not show anticancer activity by itself [23,24]. We

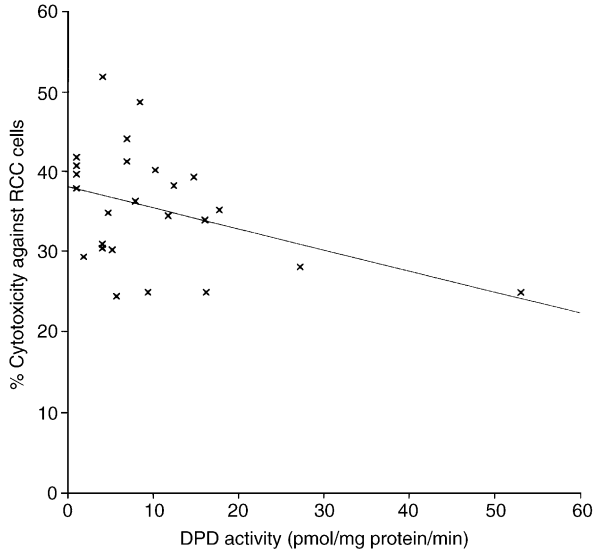

Fig. 5. Relationship between the level of DPD activity in the RCC cells and their sensitivity to 5-FU. Twenty-three primary cultured RCC cells and two RCC cell lines were used. There was an inverse correlation between the level of DPD activity in the RCC cells and their sensitivity to 5-FU (100 μM) ($n=25$, $r=0.4$, $P<0.05$ by Pearson's correlation test).

examined whether CDHP enhanced the cytotoxic activity of 5-FU against RCC cells. Although treatment with CDHP had no cytotoxic effect on primary cultured RCC cells, CDHP significantly augmented the cytotoxic activity of 5-FU (Table 2). 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 a therapeutic option to overcome the resistance of RCCs to 5-FU.

4. Discussion

In the present study, we demonstrated that DPD activity was downregulated in RCC compared with normal kidney, and that the level of DPD activity was inversely correlated with a more progressive stage of disease and increased grade of RCC. Furthermore, clear cell RCC had a lower DPD activity, compared with papillary RCC. The prognosis in patients with papillary RCC has been reported to be better than that in patients with clear RCC [25,26]. Since DPD activity decreases in parallel with the increase in the cancer growth rate and reincorporation of pyrimidine bases into RNA and DNA through the salvage pathway [27,28], RCC with a low DPD activity may be associated with an aggressive phenotype. Although we report on a small number of patients, our current data suggest that a low DPD activity may be related to the malignant potential of RCC and may be a poor prognostic factors in RCC.

Table 2
Enhancement of the sensitivity of RCC cells to 5-FU using CDHP

Drugs	% Cytotoxicity (mean±S.D.)[a]
5-FU (10 μM)	20.0±8.0
CDHP	1.5±1.5
5-FU (10 μM)+CDHP	55.7±15.8[b]
5-FU (100 μM)	38.4±8.6
5-FU (100 μM)+CDHP	75.3±9.6[b]

CDHP, 5-chloro-2,4-dihydroxypyridine.

[a] The direct cytotoxic effect of 5-FU (10–100 μM) with or without CDHP (10 μM) on primary cultured RCC cells was assessed by a 1-day MTT assay. The RCC cells were derived from 21 patients with RCC.

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

It has been reported that there is a circadian variability in DPD activity measured in blood lymphocytes from humans with a 2-fold ratio between maximum and minimum activity [5]. Due to feasibility problems, no data are available on DPD circadian variability in other human tissues. Therefore, 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 RCC patients undergoing radical nephrectomy were sampled at the same time, but at different times of the day for each patient. Nevertheless, the level of DPD activity in normal lesions was higher than that in tumoral lesions. These findings suggest that the circadian variation of DPD activity in renal tumoral and non-tumoral tissues may not be critical in this study.

DPD activity in normal kidney samples (range: 4.0–62.4 pmol/mg protein/min) was 2-fold higher than that in RCC samples (range: 1.5–53.2 pmol/mg protein/min). The ratio of normal/cancer DPD activity in patients with RCC is different from those reported in patients with head and neck cancer, and colorectal cancer, for which the median ratios were 0.96 (range of DPD activity in cancer: 13–193 pmol/mg protein/min) and 1.32 (range of DPD activity in cancer: 28–207 pmol/mg protein/min), respectively [12,13]. This high ratio of normal/cancer DPD activity in RCC may contribute to the favourable differential between anticancer effect and the adverse effects of 5-FU. Thus, a higher degree of 5-FU degradation may occur in normal kidney, compared with that in RCC.

The present study demonstrated that DPD activity in RCC cells was inversely correlated with their sensitivity to 5-FU, and that CDHP, a inhibitor of DPD activity, 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 was used in combination with 5-FU [29,30]. 5-benzyloxybenzyluracil, a strong DPD inhibitor, increased 5-FU cytotoxicity in human colon tumour xenografts [8]. Another promising DPD inhibitor, ethynyluracil, improved the efficacy and therapeutic index of

5-FU [7]. Altogether, these findings suggest that FU catabolism in RCC cells may be one of determinant factors for 5-FU responsiveness, and justify the clinical use of specific DPD inhibitors in combination with 5-FU for RCC, although the resistance to 5-FU is 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 [22,23]. 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 oral fluoropyrimidines. S-1 is now used clinically against gastric cancer, and the efficacy is good. The current findings suggest that S-1 may be a potential anticancer agent against RCC.

Many experimental studies performed on various cancers have demonstrated that either overexpression of TS protein or high TS activity is associated with 5-FU resistance [31,32]. In addition, the level of TS activity predicted the response to 5-FU-based chemotherapy [33,34]. These studies have shown that the lower the TS activity is, the greater the response rate to 5-FU-containing chemotherapy achieved. Since TS is the main target enzyme for 5-FU, the measurement of TS activity, as well as DPD activity, may be necessary for evaluating the efficacy of 5-FU-based chemotherapy.

We used a TLC assay to measure DPD activity. The DPD enzyme activity detected by TLC assay is correlated with DPD gene expression by utilising semiquantitative polymerase chain reaction (PCR) analysis [35]. The DPD activity is also associated with the expression of DPD protein determined by Western blotting analysis [36]. In addition, preliminary experiments demonstrated that the DPD activity is related to the expression of DPD protein measured by the enzyme-linked immunosorbent assay. These findings suggest that the DPD activity correlates with the expression of DPD mRNA and protein.

In conclusion, the current study demonstrated that the DPD activity in RCC decreased in parallel with an increased histological stage and grade, and that the sensitivity of RCC to 5-FU was inversely correlated with the DPD activity and was enhanced by a DPD inhibitor. These findings suggest that the assessment of DPD activity may be useful both in the management and treatment of RCC. Since the level of DPD activity may be associated with the malignant potential of RCC, an accurate prediction of prognosis using this marker may help to select patients for more intensive surgical, immunotherapeutic or chemotherapeutic approaches. Furthermore, a combination of 5-FU and DPD inhibitors may be effective against RCC. However, further analysis will be needed on the regulatory effects of DPD activity and the in vivo combined effects of 5-FU and DPD inhibitors.

Acknowledgements

This work was supported, in part, by a 2002 Grant-in-Aid from the Japanese Urological Association and a Grant-in-Aid from the Japanese Ministry of Education, Science and Culture (No. 14657410).

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