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Increased dihydropyrimidine dehydrogenase activity associated with mild toxicity in patients treated with 5-fluorouracil and leucovorin

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ABSTRACT

Dihydropyrimidine dehydrogenase (DPD) plays a pivotal role in the metabolism of 5FU. The prognostic significance of DPD activity in peripheral blood mononuclear (PBM) cells and buccal mucosa cells with respect to toxicity was investigated in 44 patients treated with 5FU-leucovorin. Grade III/IV haematological and grade III/IV gastrointestinal toxicity were observed in 25% and 21% of the patients, respectively. No association was observed between the DPD activity in buccal mucosa cells and toxicity. In contrast, the mean DPD activity in PBM cells proved to be increased in patients experiencing grade I/II neutropenia when compared to patients without neutropenia and those suffering from grade III/IV neutropenia ($P = 0.002$). Patients with a high-normal DPD activity proved to be at risk of developing mild toxicity upon treatment with 5FU-leucovorin, suggesting an important role of DPD in the aetiology of toxicity associated with catabolites of 5FU.

1. Introduction

5-Fluorouracil (5FU) is one of the most frequently prescribed chemotherapeutic drugs for the curative and palliative treatment of patients with cancers of the gastrointestinal tract, breast and head and neck. The treatment of patients with stage III colorectal cancer with adjuvant 5FU-based chemotherapy has increased the likelihood of 5-year overall survival from 51% to 64%.[1] Nevertheless, approximately 40% of these patients will still die from metastatic disease, despite surgery and adjuvant chemotherapy while 5FU-induced toxicity can be profound.

An analysis involving 974 patients with colorectal cancer treated with 5FU/leucovorin, administered according to the Mayo Clinic regimen, showed that grade III or grade IV neutropenia, stomatitis and diarrhoea occurred in 26%, 14% and 13% of the patients, respectively.[2] Severe stomatitis, especially its ulcerative form, increased the risk of systemic infections, sepsis and even mortality in immunocompromised patients.[3] Severe pain interferes with the quality of life and food intake,

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and often requires cessation of the therapy.[3] Therefore, the identification of genetic factors predisposing patients to the development of severe 5FU-associated toxicity is increasingly being recognised as an important field of study.

The cytotoxic effect of 5FU has been ascribed to the formation of fluoropyrimidine nucleotides, which interfere with the synthesis and stability of RNA, DNA and cellular membranes.[4,5] Opposing the activation of 5FU *via* the anabolic pathways are the enzymes of the pyrimidine degradation pathway. Dihydropyrimidine dehydrogenase (DPD) catalyses the conversion of 5FU to fluoro-5,6-dihydrouracil (FUH_2), which is the initial and rate-limiting step in the catabolism of 5FU. FUH_2 can be further degraded to fluoro-β-ureidopropionate (FUPA) and subsequently to fluoro-β-alanine (FBAL) by dihydropyrimidinase and β-ureidopropionase, respectively.

It has been shown that DPD plays a pivotal role in the metabolism of 5FU.[6,7] Because more than 80% of the administered 5FU is catabolised by DPD, patients with a complete or partial DPD deficiency have a strongly reduced capacity to degrade 5FU.[8,9] Owing to the fact that 5FU has a relatively narrow therapeutic index, those patients with a complete or partial DPD deficiency have an increased likelihood of suffering from severe and sometimes even lethal drug-induced toxicity.[10–12]

The activity of DPD can be detected in a variety of tissues but the liver is the main organ responsible for the catabolism of 5FU.[13,14] Since the activity of DPD in normal liver correlates well with that of PBM cells, the latter have been used as a surrogate for total body DPD activity.[15] A number of studies have suggested that the intra-tumoural levels of DPD may be an important prognostic factor of response to 5FU.[7] Reasoning along these lines, it is conceivable that a low level of DPD in buccal mucosa cells would be indicative for patients with an increased risk of developing stomatitis. To date, no studies have been reported regarding the role of DPD in buccal mucosa and 5FU-associated stomatitis. In this study, we have therefore investigated the prognostic significance of DPD activity in PBM cells and buccal mucosa cells of cancer patients treated with 5FU/leucovorin, administered according to the Mayo Clinic regimen, with respect to toxicity in general and haematological toxicity and stomatitis in particular.

2. Patients and methods

2.1. Patients

The study group consisted of 44 cancer patients who had not received previous chemotherapy and were treated in the Academic Medical Center in Amsterdam, between 1998 and 2004, with 5FU/leucovorin administered according to the Mayo Clinic regimen (20 mg/m^2 leucovorin followed by 425 mg/m^2 5FU, administered as an i.v. bolus on days 1–5 every 28 days).[2] None of these patients received chemotherapy at the time of blood sampling and collection of buccal mucosa cells for determination of the DPD activity. All samples were obtained between 10 am and 12 pm. The toxicity experienced by the patients upon subsequent treatment with 5FU-based chemotherapy was graded in accordance with the Common Toxicity Criteria. Informed consent was obtained from all patients and healthy volunteers (n = 11) for collection of the buccal mucosa cells.

2.2. Isolation of PBM cells and buccal mucosa cells

PBM cells were isolated from 15 ml EDTA-anticoagulated blood by centrifugation over lymphoprep and the cells from the interface were collected and treated with ice-cold NH_4Cl to lyse the contaminating erythrocytes, as described before.[16]

Prior to the collection of the buccal mucosa cells, the patients were asked to rinse their mouth with water. The inner cheek was gently scraped 10 times with a plastic knife followed by rinsing of the mouth with 15–20 ml of phosphate-buffered saline (PBS). The mouthwash was expectorated into a 50 ml centrifuge tube and the procedure was repeated to collect the buccal mucosa cells from the other inner cheek. After the addition of 10–20 ml of PBS to the combined mouthwashes, the buccal cells were collected by centrifugation (1600g, 10 min). The cell pellet was resuspended in ≈1 ml PBS and 20 μl was saved for assessment of the cell viability using the Trypan Blue exclusion method. The remaining cell suspension was centrifuged at 13,000g for 10 s. The supernatant was discarded and the pellet was frozen in liquid nitrogen and stored at –80 °C until further analysis.

2.3. Determination of DPD activity

The activity of DPD was determined in a reaction mixture containing 35 mM potassium phosphate (pH 7.4), 2.5 mM $MgCl_2$, 1 mM dithiothreitol, 250 μM NADPH and 25 μM [^{14}C]-thymine.[16] Separation of radiolabelled thymine from radiolabelled dihydrothymine was performed isocratically (50 mM NaH_2PO_4 (pH 4.5) and 7.5% (v/v) methanol) at a flow rate of 1 ml/min by HPLC on a reversed-phase column (Aqua 125A C18, 250 × 4.6 mm, 5 μm particle size, Phenomenex, Torrance, CA) and a guard column (Security guard C18, 4 mm × 3.0 mm ID, Phenomenex, Torrance CA, USA) with online detection of the radioactivity. Protein concentrations were determined with a copper-reduction method using bicinchoninic acid, essentially as described by Smith et al.[17]

2.4. Statistics

Analysis to determine whether the DPD activity in PBM cells and buccal mucosa cells followed a Normal distribution pattern was performed using the Kolmogorov–Smirnov test. Association of the DPD activity in PBM cells and the degree of toxicity was performed with a one-way ANOVA combined with a *post hoc* test using least-significant difference. The association of the DPD activity in buccal mucosa cells and the degree of toxicity was performed with a Kruskal–Wallis test. Comparison of the DPD activity between two groups was performed using the two sample Student's t-test. The correlation between the DPD activity in PBM cells and buccal mucosa cells was analysed by determination of the Pearson correlation coefficient. The correlation between the DPD activity and the viability of buccal mucosa cells was analysed by means of Spearman's rank correlation. The level of significance was set *a priori* at $P \leqslant 0.05$. Analyses were performed using the Statistical Package for the Social Sciences (SPSS), version 12.0.2 (SPSS Inc., IL, USA).

3. Results

3.1. Patients characteristics and clinical presentation

The characteristics of the patients are summarised in Table 1. The majority of the patients (70%) were suffering from colon cancer followed by sigmoid (16%), rectal (11%) and breast cancer (2%). The various types of toxicities encountered in the patients upon treatment with 5FU-leucovorin, administered according to the Mayo Clinic regime, are shown in Tables 2 and 3. Grade III/IV haematological and grade III/IV gastrointestinal toxicity were observed in 25% and 21% of the patients, respectively. The most prevalent type of grade III/IV haematological toxicity was neutropenia. Patients experiencing grade III/IV gastrointestinal toxicity mainly suffered from diarrhoea and stomatitis. In the total group of patients, grade III toxicity was observed in 10 patients (22%) whereas 8 patients (18%) suffered from grade IV toxicity (Table 3).

3.2. DPD activity in PBM cells and buccal mucosa cells

The distribution of the DPD activity in PBM cells from cancer patients followed a normal or Gaussian distribution and it ranged from 4.2 to 16.0 nmol/mg/h (Fig. 1A). In contrast, the distribution of the DPD activity in buccal mucosa cells proved to be skewed and ranged from 0.06 to 6.2 nmol/mg/h (Fig. 1B). No correlation was observed between DPD activity in PBM cells and that of buccal mucosa cells ($P = 0.71$). The large variation in the DPD activity in the buccal mucosa cells was not due to the assay itself as the intra-assay coefficient of variation (CV) and inter-assay CV were 2.4% and 4.2%, respectively. The DPD activity in buccal mucosa cells from cancer patients (1.9 ± 1.5 nmol/mg/h, $n = 24$) was comparable to that observed in healthy volunteers (1.7 ± 1.0 nmol/mg/h, $n = 11$). Furthermore, the average viability of the isolated buccal mucosa cells was comparable in cancer patients (36% ± 13%, $n = 19$) and healthy volunteers (39 ± 20%, $n = 11$). No correlation was observed between the DPD activity and the viability of the buccal mucosa cells from both cancer patients and healthy volunteers ($P = 0.24$). The mean DPD activity in PBM cells and buccal mucosa cells was comparable in men and women (Table 1).

Table 1 – Patient characteristics

	Patient group ($n = 44$)	Men	Women
Age (year)			
Mean ± SD	57 ± 10	57 ± 12	56 ± 9
Range	31–78	31–78	35–68
Gender		26	18
Cancer localisation			
Colon	31	18	13
Sigmoid	7	5	2
Rectal	5	3	2
Breast	1	0	1
DPD activity (nmol/mg/h)			
PBM cells			
Mean ± SD	9.6 ± 2.6	9.7 ± 2.5	9.5 ± 2.7
Range	4.2–16.0	4.9–13.4	4.2–16.0
Buccal mucosa cells			
Mean ± SD	1.9 ± 1.5 ($n = 24$)	1.7 ± 1.2 ($n = 13$)	2.2 ± 1.9 ($n = 11$)
Range	0.06–6.2	0.06–3.9	0.21–6.2

Table 2 – Toxicity profile of patients treated with 5FU/leucovorin

Toxicity	0	I	II	III	IV
Haematological					
Neutropenia	27	5	1	5	6
Trombocytopenia	43	1	0	0	0
Gastrointestinal					
Nausea	23	14	6	1	0
Vomiting	37	6	1	0	0
Diarrhoea	20	10	8	3	3
Stomatitis	21	12	4	6	1
Anorexia	31	10	2	0	1
Others	41	1	2	0	0
Flu-like symptoms					
Fever	40	1	1	2	0
Malaise	35	6	3	0	0
Fatigue	38	3	2	1	0
Others					
Dermatological	37	7	0	0	0
Neurological	43	1	0	0	0
Alopecia	41	2	0	1	0
Hand-foot syndrome	38	3	2	1	0
Others	37	6	1	0	0

The figures represent the number of patients suffering from a particular type of toxicity (graded according to the Common Toxicity Criteria). Individual patients can experience multiple types of toxicities.

Table 3 – Clinical presentation of patients treated with 5FU/leucovorin

	0	I	II	III	IV	III/IV (%)
Haematological	26	6	1	5	6	25
Gastrointestinal	6	17	12	6	3	21
Flu-like symptoms	27	9	5	3	0	7
Others	26	14	2	2	0	5
Overall toxicity	4	12	10	10	8	41

The figures represent the number or percentage of patients suffering from a particular type of toxicity (graded according to the Common Toxicity Criteria).

3.3. DPD activity and toxicity

Table 4 shows the DPD activity in buccal mucosa cells of patients experiencing no toxicity, grade I/II toxicity or grade III/IV toxicity. A large variation and range of DPD activity was observed in buccal mucosa cells in these three groups and the DPD activity in buccal mucosa cells was not associated with haematological, gastrointestinal, flu-like symptoms or other types of toxicities. Furthermore, no correlation was observed with specific types of gastrointestinal toxicities such as diarrhoea or stomatitis.

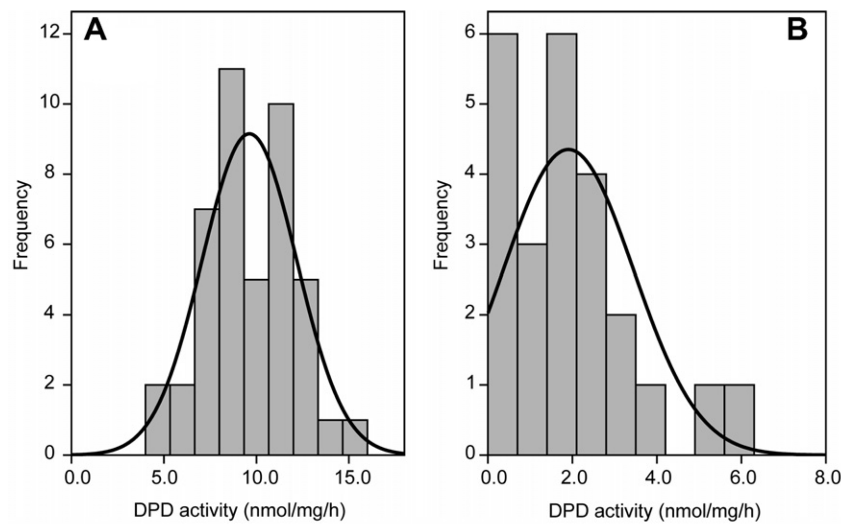

Fig. 1 – Histograms of the DPD activity. Panel A shows the distribution of the DPD activity in PBM cells. The distribution of the DPD activity in buccal mucosa cells is shown in panel B. The lines represent the normal distribution.

Table 4 – DPD activity in buccal mucosa cells of patients and 5FU-associated toxicity

Toxicity	Grade 0 (*n*)	Grades I–II (*n*)	Grades III–IV (*n*)	*P*
Haematological	2.1 ± 1.8 (15)	2.1 ± 1.2 (4)	1.3 ± 1.0 (5)	0.60
Gastrointestinal	1.6 ± 0.9 (4)	1.9 ± 1.4 (14)	2.2 ± 2.2 (6)	0.81
Flu-like symptoms	2.0 ± 1.8 (16)	1.9 ± 1.1 (6)	1.5 ± 1.4 (2)	0.93
Others	1.8 ± 1.5 (17)	2.4 ± 1.8 (6)	1.4 (1)	0.67

Table 5 – DPD activity in PBM cells of patients and 5FU-associated toxicity

Toxicity	Grade 0 (*n*)	Grades I–II (*n*)	Grades III–IV (*n*)	*P*
Haematological				
Neutropenia	9.0 ± 2.0 (27)	12.9 ± 1.7 (6)	9.3 ± 2.9 (11)	0.002
Trombocytopenia	9.5 ± 2.5 (43)	13.3 (1)	n.p.	
Gastrointestinal				
Nausea	9.5 ± 2.5 (23)	9.8 ± 2.7 (20)	8.7 (1)	0.85
Vomiting	9.9 ± 2.6 (37)	8.2 ± 2.1 (7)	n.p.	0.11
Diarrhoea	9.9 ± 2.3 (20)	9.7 ± 2.8 (18)	8.6 ± 2.8 (6)	0.58
Stomatitis	9.8 ± 2.2 (21)	9.6 ± 2.8 (16)	9.0 ± 3.2 (7)	0.78
Anorexia	9.7 ± 2.5 (31)	9.1 ± 2.7 (12)	13.3 (1)	0.27
Others	9.6 ± 2.6 (41)	9.7 ± 0.8 (3)	n.p.	0.96
Flu-like symptoms				
Fever	9.6 ± 2.6 (40)	8.8 ± 0.3 (2)	10.7 ± 3.5 (2)	0.76
Malaise	9.6 ± 2.4 (35)	9.9 ± 3.2 (9)	n.p.	0.76
Fatigue	9.4 ± 2.4 (38)	10.6 ± 3.7 (5)	13.3 (1)	0.21
Others				
Dermatological	9.5 ± 2.5 (37)	10.0 ± 3.0 (7)	n.p.	0.65
Neurological	9.7 ± 2.6 (43)	8.0 (1)	n.p.	
Alopecia	9.5 ± 2.4 (41)	8.7 ± 2.6 (2)	16.0 (1)	
Hand-foot syndrome	9.5 ± 2.4 (38)	10.8 ± 3.7 (5)	7.1 (1)	0.37
Others	9.6 ± 2.6 (37)	9.5 ± 2.6 (7)	n.p.	0.9

The DPD activity (nmol/mg/h) is expressed as the mean ± SD.
n, the number of patients.
n.p., no patients.

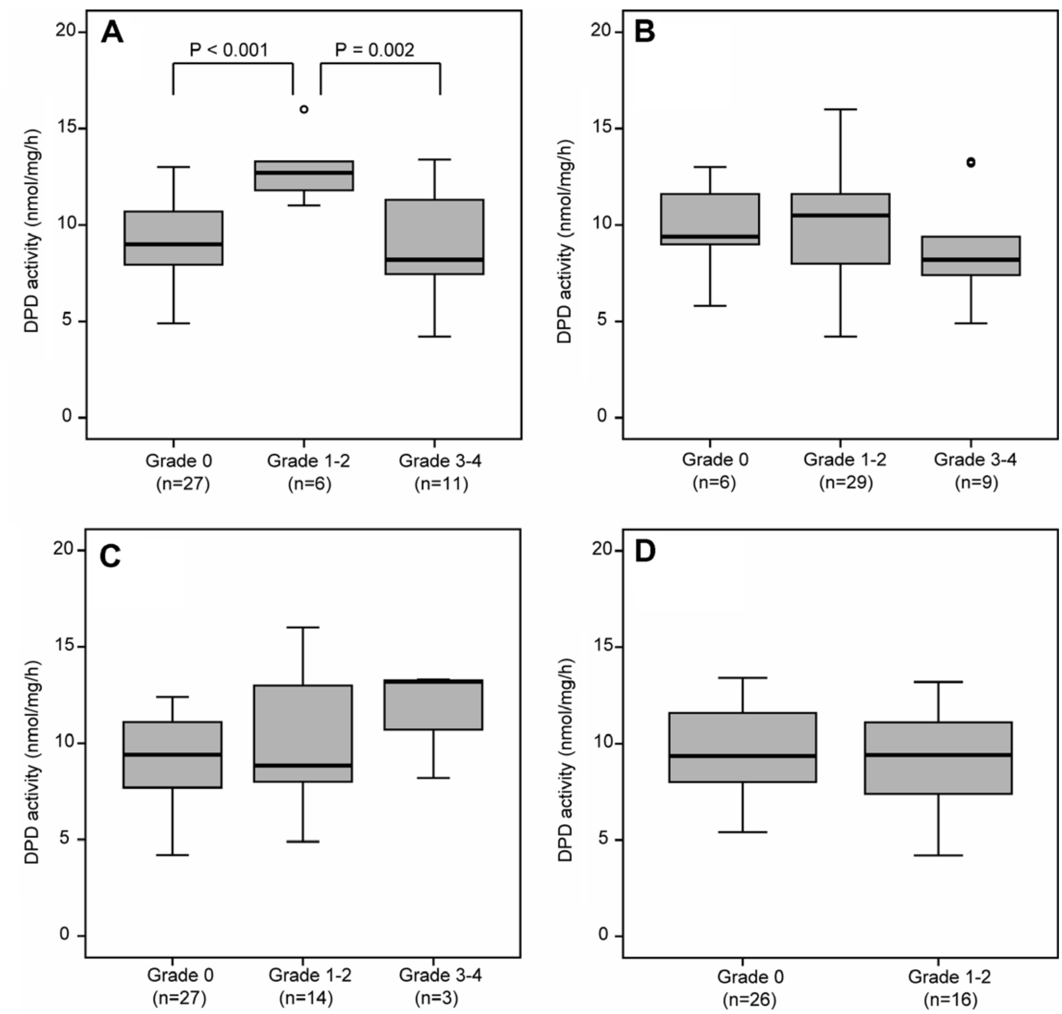

Fig. 2 – Box plots of the DPD activity in patients treated with 5FU-leucovorin. The top, bottom and line through the middle of a box correspond to the 75th percentile, 25th percentile and 50th percentile, respectively. The whiskers on the bottom extend from the 5th percentile and top 95th percentile. The open circles represent outliers. The distribution of the DPD activity is indicated for patients with haematological toxicity (panel A), gastrointestinal toxicity (panel B), Flu-like symptoms (panel C) and other types of toxicity (panel D).

In contrast, altered DPD activity in PBM cells proved to be associated with haematological toxicity (Table 5). The mean DPD activity in patients experiencing grade I/II neutropenia was significantly higher compared to patients without neutropenia ($P < 0.001$) and those suffering from grade III/IV neutropenia ($P = 0.002$) (Fig. 2A). The DPD activity in PBM cells was not associated with gastrointestinal, flu-like symptoms or other types of toxicities (Fig. 2). Furthermore, no significant differences were observed in DPD activity and the severity of diarrhoea or stomatitis (Table 5). However, it should be noted that 2 out of 9 patients suffering from grades 3–4 gastrointestinal toxicity possessed a high DPD activity (Fig. 2B).

4. Discussion

In this study, we have investigated the relationship between the DPD activity in PBM cells and buccal mucosa cells and the degree of toxicity experienced by cancer patients treated with 5FU-leucovorin. The most common treatment-related adverse events encountered in the patients treated with 5FU-leucovorin were haematological and gastrointestinal toxicities. Grade III/IV neutropenia was observed in 25% of the patients and grade III/IV diarrhoea and stomatitis was observed in 14% and 16% of the patients, respectively. The types of toxicities as well as the percentage of patients experiencing severe toxicity is comparable to that observed for 983 patients treated with 5FU/LV according to the Mayo Clinic regimen.[2,18] In those patients, grade III/IV neutropenia, diarrhoea and stomatitis were observed in 26%, 13% and 14% of the patients, respectively.[2]

In our study, the DPD activity in PBM cells from cancer patients followed a normal or Gaussian distribution and ranged from 4.2 to 16.0 nmol/mg/h. In contrast, a large 100-fold range in the activity of DPD was observed for buccal mucosa cells. In this respect, it should be noted that the DPD activity is usually lower in proliferating immature cells compared to that observed in non-proliferating normal cells.[19,20] Thus, the presence of both immature and mature buccal mucosa cells might underlie some of the variation observed in the DPD activity.[21] Furthermore, a low cell viability was observed for the buccal mucosa cells from healthy volunteers and patients, which is in line with that observed by others.[21,22] In general, cell viability has been shown to be low in epithelial tissues with terminally differentiated cell populations and a high renewal rate.[22]

5FU has a relatively narrow therapeutic index and a strong correlation has been described between exposure to 5FU and both haematological and gastrointestinal toxicities.[23] One of the dose limiting toxicities of 5FU-based regimens is stomatitis caused by damage of the rapidly growing cells of the tissue of the oral cavity. The fact that only a very low activity of DPD could be detected in buccal mucosa cells and yet no correlation was observed between DPD activity in buccal mucosa cells and the development of stomatitis suggests that the metabolism of 5FU in these cells is not significantly affected by the level of DPD. In addition, the large variation in DPD activity in buccal mucosa cells might also be partly responsible for the fact that no association was observed between the DPD activity and toxicity.

For DPD activities within the normal range, conflicting results have been published as to whether a correlation exists between the DPD activity in PBM cells and the clearance of 5FU.[24–27] Patients with a partial DPD deficiency have an increased risk of developing grade IV neutropenia.[11,28] A conspicuous finding was, therefore, the increased DPD activity in PBM cells of patients experiencing mild grade I/II neutropenia when compared to the DPD activity in PBM cells of patients without neutropenia and those suffering from grade III/IV neutropenia. In this respect, it is worthwhile to note that the downstream catabolites of 5FU have been associated with toxicity. A patient with a partial dihydropyrimidinase deficiency and thus a decreased capacity to degrade FUH_2 suffered from severe toxicity, including leucopenic fever.[29] In rats, FUH_2 and FBAL attenuated the antitumour activity and increased the toxicity of 5FU.[30,31] Thus, mild neutropenia (grade I/II) might be associated with increased concentrations of the catabolic products of 5FU and therefore, an increased activity of DPD. In contrast, severe neutropenia (grade III/IV) might be caused by increased levels of fluoropyrimidine nucleotides, the anabolic products of 5FU, and thus a decreased activity of DPD.

A similar phenomenon might explain the apparent lack of a clear association between the severity of gastrointestinal toxicity and the DPD activity in PBM cells. A large variation in DPD activity was observed in patients suffering from grades 3–4 gastrointestinal toxicity and 2 out of 9 patients possessed a high DPD activity. A pharmacokinetic analysis showed that a positive correlation existed between the AUC of FBAL and grades 3–4 diarrhoea.[32] In addition, the plasma levels of FBAL correlated with the DPD activity in PBM cells.[33] Thus, it is conceivable that both patients with a decreased DPD activity or an increased DPD activity, resulting in increased levels of fluoropyrimidine nucleotides and downstream catabolites of 5FU, respectively, are prone to develop severe gastrointestinal toxicity.

Whereas the role of adjuvant treatment is well established in stage III disease, the value of post-operative 5FU-based therapy after resection of stage II colon cancers remains controversial.[2] The identification of patients who are prone to develop 5FU-associated toxicity would allow either dose-adaptation or the application of alternative agents. To date, ample evidence has been provided that in case of a deficiency of DPD, profound alterations in the metabolism of 5FU can be expected with an increased likelihood of developing severe toxicity.[6–10] In this paper, we showed that even patients with a high-normal DPD activity proved to be at risk of developing mild haematological toxicity upon treatment with 5FU-based chemotherapy, thus further strengthening the important role of DPD in the aetiology of 5FU-associated toxicity.

Conflict of interest statement

The authors have no conflicts of interests to disclose.

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