Clinical Implications of Dihydropyrimidine Dehydrogenase (DPD) Deficiency in Patients with Severe 5-Fluorouracil-associated Toxicity: Identification of New Mutations in the *DPD* Gene

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ABSTRACT

Dihydropyrimidine dehydrogenase (DPD) is the initial and rate-limiting enzyme in the catabolism of 5-fluorouracil (5FU), and it is suggested that patients with a partial deficiency of this enzyme are at risk for developing a severe 5FU-associated toxicity. To evaluate the importance of this specific type of inborn error of pyrimidine metabolism in the etiology of 5FU toxicity, an analysis of the DPD activity, the *DPD* gene, and the clinical presentation of patients suffering from severe toxicity after the administration of 5FU was performed. Our study demonstrated that in 59% of the cases, a decreased DPD activity could be detected in peripheral blood mononuclear cells. It was observed that 55% of patients with a decreased DPD activity suffered from grade IV neutropenia compared with 13% of patients with a normal DPD activity ($P = 0.01$). Furthermore, the onset of toxicity occurred, on average, twice as fast in patients with low DPD activity as compared with patients with a normal DPD activity (10.0 ± 7.6 *versus* 19.1 ± 15.3 days; $P < 0.05$). Analysis of the *DPD* gene of 14 patients with a reduced DPD activity revealed the presence of mutations in 11 of 14 patients, with the splice site mutation IVS14+1G→A being the most abundant one (6 of 14 patients; 43%). Two novel missense mutations 496A→G (M166V) and 2846A→T (D949V) were detected in exon 6 and exon 22, respectively. Our results demonstrated that at least 57% (8 of 14) of the patients with a reduced DPD activity have a molecular basis for their deficient phenotype.

INTRODUCTION

5FU[2] remains one of the most frequently prescribed chemotherapeutic drugs for the treatment of cancers of the gastrointestinal tract, the breast, and the head and neck. It has been shown that a long-term continuous i.v. infusion of 5FU is superior to bolus injections of 5FU in terms of response rate, although only a small increase in median survival was observed (1). In addition, a relationship between the 5FU dose intensity and the therapeutic response, as well as toxicity, has been noted (2, 3). To exert its cytotoxicity, 5FU must first be metabolized to the nucleotide level. Although the cytotoxic effects of 5FU are probably mediated directly via the anabolic pathways, the catabolic route plays a significant role because >80% of the administered 5FU is catabolized by DPD (4). In line with this phenomenon is the recent finding that tumors expressing low levels of DPD mRNA and DPD activity show a significantly better response to 5FU than those tumors with a high mRNA level and DPD activity (5, 6). Most importantly, a low intratumoral expression level of the *DPD* gene appears to be associated with a much longer survival period for patients (6).

Because 5FU has a relatively narrow therapeutic index, toxicity increases as the dose is increased, resulting in escalated plasma levels of the drug (2, 3). A correlation has been observed between the pretreatment activity of DPD in PBM cells and the systemic clearance of 5FU in cancer patients (7). In this light, a pharmacogenetic disorder has recently been described concerning cancer patients with a complete or partial deficiency of DPD suffering from a severe or even life-threatening toxicity after the administration of 5FU. It was shown that a number of these patients were genotypically heterozygous for a mutant *DPD* allele (8–11). On the basis of a population analysis of the DPD activity, the frequency of heterozygotes has been estimated to be as high as 3% (7). Such patients might be at risk of developing severe toxicity after the administration of 5FU.

Thus far, 17 variant *DPD* alleles have been identified in pediatric patients suffering from a complete or nearly complete deficiency or in tumor patients with a decreased DPD activity (11–14). Recently, Collie-Duguid *et al.* (11) suggested that the known variant *DPYD* alleles do not entirely explain the poly-

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[2] The abbreviations used are: 5FU, 5-fluorouracil; DPD, dihydropyrimidine dehydrogenase; PBM, peripheral blood mononuclear; AUC, area under the curve.

Table 1 PCR primers for determination of intron-exon boundaries of the *DPD* gene

Exon	Gene-specific primer 1	Gene-specific primer 2
4[a]	5′-ATGCCTGAAATGTGCAGATGCC-3′	5′-CAGAAGAGCTGTCCAACTAATCTTG-3′
9[a]	5′-GATGCCATCTTCCAAGGCCTG-3′	5′-GCAGGACCAGGGGTTTTATACATC-3′
12[a]	5′-TGTGAATGTACCAAGAAGCTTGCT-3′	5′-CTGCAAATACCCATGCTTCACTAGT-3′
12[a]	5′-CAGAAGTAGATCCAGAAACTATGC-3′	5′-CATGGGTATTTGCAGGTGGTGATG-3′
15[b]	5′-CTCAGACTTCTTGGCAAGTTCC-3′	5′-GTCCAGTCATTTTTATTGTAACTGC-3′
21[a]	5′-GCCAAGTTTTGGACCTTATCTGG-3′	5′-AATGTAGCTTTTCACCACTTAAGAG-3′

[a] For determination of the 5′ donor site.
[b] For determination of the 3′ acceptor site.

Table 2 Oligonucleotides used for genomic PCR of DPD sequences

Exon	Primers	Primer sequences	PCR temperature (°C)	Fragment (bp)
1	DPDEIF	5′-GCTGTCACTTGGCTCTCT-3′	50	184
	DPD111R1	5′-CACCTACCCGCAGAGCA-3′		
2	M13DPD108F	5′-M13F-GTGACAAAGTGAGAGAGACCGTGTC-3′[a]	65	321
	M13DPD109R	5′-M13R-GCCTTACAATGTGTGGAGTGAGG-3′[b]		
3	DPD112F	5′-GAATGCTACCCAATTAAAGTGG-3′	55	267
	DPD113Rd	5′-CCTACCACCATCCTGTGACTG-3′		
4	DPD96F	5′-GGTAGAAAATAGATTATCTC-3′	50	245
	DPD4R	5′-GATTTGCTAAGACAAGCTG-3′		
5	DPD94F	5′-GTTTGTCGTAATTTGGCTG-3′	60	284
	DPD95R	5′-ATTTGTGCATGGTGATGG-3′		
6	DPD92F	5′-GAGGATGTAAGCTAGTTTC-3′	52	357
	DPD93R1	5′-CCATTTGTGTGCGTGAAGTTC-3′[a]		
7	DPD90F	5′-GTCCTCATGCATATCTTGTGTG-3′[b]	52	360
	DPD121R	5′-GCTTCTGCCTGATGTAGC-3′		
8	DPD122F	5′-GCCCCACATCGTGCTATGAACA-3′	57	459
	DPD91R1	5′-GTCTGAAGGCAGTCATTCTGG-3′		
9	M13DPD88F	5′-M13F-CCCTCCTCCTGCTAATG-3′[a]	52	278
	M13DPD9R-2	5′-M13R-GAACAATGTGCTGCTGAG-3′[b]		
10	DPD86F	5′-GAGAGTGACACTTCATCCTGG-3′	60	342
	DPD87R	5′-CTGTTGGTGTACAACTCC-3′		
11	DPD84	5′-ACTGGTAACTGAAACTCAG-3′	52	442
	DPD85R4	5′-CAATTCCCTGAAAGCTAG-3′		
12	DPD12F	5′-TTCCTGTATGTGAGGTGTA-3′	55	453
	DPD83R1	5′-GAAGCACTTATCCATTGG-3′		
13	DPD70F	5′-CGGATGACTGTGTTGAAGTG-3′	55	440
	DPD71R	5′-TGTGTAATGATAGGTCGTGTC-3′		
14	DPD14F	5′-TCCTCTGCAAAAATGTGAGAAGGGACC-3′	55	370
	DPD14R	5′-TCACCAACTTATGCCAATTCTC-3′		
15	M13DPD15F	5′-M13F-TATCTTTGTGTACAACTGGA-3′[a]	52	391
	M13DPD119R	5′-M13R-TGTGAAATCCAAGGGACC-3′[b]		
16	DPD116F	5′-AACGGTGAAAGCCTATTGG-3′	52	223
	DPD117R	5′-TAGTAACTATCCATACGGGGG-3′		
17	DPD114F	5′-CACGTCTCCAGCTTTGCTGTTG-3′	60	238
	DPD115R	5′-CGGGCAACTGATTCAAGTCAAG-3′		
18	DPD98F	5′-TGGGATGTGAGGGGGTGAATG-3′	60	248
	DPD99R	5′-TTCAGCAACCTCCAAGAAAGCCAC-3′		
19	DPD100F	5′-TGTCCAGTGACGCTGTCATCAC-3′	55	300
	DPD80R	5′-CATTGCATTTGTGAGATGGAG-3′		
20	DPD78F	5′-GAGAAGTGAATTTGTTTGGAG-3′	55	424
	DPD79R1	5′-CACAGACCCATCATATGGCTG-3′		
21	M13DPD76F	5′-M13F-TCTGACCTAACATGCTTC-3′[a]	48	264
	M13DPD21R	5′-M13R-CCAGTAAAGTAGGCATAC-3′[b]		
22	DPD74F	5′-GAGCTTGCTAAGTAATTCAGTGGC-3′	60	291
	DPD75R	5′-AGAGCAATATGTGGCACC-3′		
23	DPDASPF	5′-GGGGACAATGATGACCTATGTGG-3′	60	269
	DPDASPR	5′-GGTGACATGAAAGTTCACAGCAAC-3′		

[a] Sequence M13F, TGT-AAA-ACG-ACG-GCC-AGT.
[b] Sequence M13R, CAG-GAA-ACA-GCT-ATG-ACC.

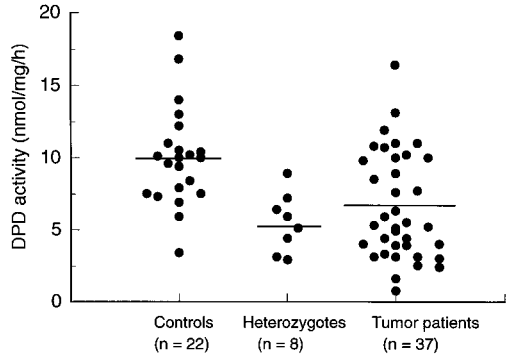

Fig. 1 DPD activity in controls, obligate heterozygotes, and tumor patients. The mean DPD activities are indicated by *solid lines*.

morphic DPD activity and toxic response to 5FU. A serious drawback of their study, however, is the fact that only 10 of the 23 exons of the *DPD* gene were sequenced and investigated for the presence of mutations. Thus, no sound evidence has yet been provided as to whether patients with a low DPD activity have a molecular basis for their reduced activity.

Despite the pivotal role DPD plays in the metabolism of 5FU, only a limited number of studies have been reported describing either the toxicity encountered in patients with a low DPD activity or the partial analysis of the *DPD* gene for the presence of a mutated *DPD* allele (11, 15). To date, no studies have been reported describing both the toxicity and the analysis at the entire *DPD* gene for the presence of mutations. Thus, to evaluate the importance of this specific type of inborn error of pyrimidine metabolism in the etiology of 5FU toxicity, we performed an analysis of the DPD activity, the entire *DPD* gene, and the clinical presentation of patients suffering from severe toxicity after the administration of 5FU. Our study demonstrates that in 59% of the cases, a decreased DPD activity might be responsible for the observed toxicity, with grade IV granulocytopenia being the most prevalent type of toxicity in these patients. Furthermore, a high incidence of mutations in the *DPD* gene was found, with the splice site mutation IVS14+1G→A being the most abundant one. Our results demonstrate that at least 57% (8 of 14 patients) of the patients with a reduced DPD activity have a molecular basis for their deficient phenotype.

MATERIALS AND METHODS

Chemicals. [4-^{14}C]thymine (1.85–2.22 GBq/mmol) was obtained from Moravek Biochemicals (Brea, CA). Lymphoprep (specific gravity, 1.077 grams/ml; 280 mOsm) was obtained from Nycomed Pharma AS (Oslo, Norway). LeucoSep tubes were supplied by Greiner (Frickenhausen, Germany). AmpliTaq Taq polymerase, BigDye-Terminator-Cycle-Sequencing-Ready Reaction kits, and BigDye-Primer-Cycle-Sequencing-Ready Reaction kits were supplied by Perkin-Elmer (Foster City, CA). A Qiaquick Gel Extraction kit was obtained from Qiagen (Hilden, Germany). All of the other chemicals used were of analytical grade.

Isolation of Human PBM Cells and Granulocytes. PBM cells were isolated from 15 ml of 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 previously (16). The pellet of the centrifugation step over Lymphoprep containing the granulocytes and erythrocytes was diluted with 7 ml of supplemented PBS [9.2 mM Na_2HPO_4, 1.3 mM NaH_2PO_4, 140 mM NaCl, 0.2% (w/v) BSA, 13 mM sodium citrate, and 5 mM glucose (pH 7.4)] and centrifuged at 800 × *g* at room temperature for 10 min. To lyse the erythrocytes, the pellet was resuspended in 7 ml of ice-cold ammonium chloride solution (155 mM NH_4Cl, 10 mM $KHCO_3$, and 0.1 mM EDTA) and kept on ice for 5 min. After the addition of 10 ml of ice-cold supplemented PBS, the solution was centrifuged at 250 × *g* at 4°C for 10 min. The pellet was collected and subjected to another lysis step as described above. The pellet containing the granulocytes was washed once more with supplemented PBS, and the final cell pellet was frozen in liquid nitrogen and stored at −80°C until further analysis.

Determination of the 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 DTT, 250 μM NADPH, and 25 μM [4-^{14}C]thymine (16). Separation of radiolabeled thymine from radiolabeled dihydrothymine was performed isocratically [50 mM NaH_2PO_4 (pH 4.5)] at a flow rate of 2 ml/min by high-performance liquid chromatography on a reversed-phase column (Alltima C18; 250 × 4.6 mm; 5 μm particle size; Alltech Associates Inc., Deerfield, IL) and a guard column (Supelguard LC-18-S; 5 μm particle size; 20 × 4.6 mm; Supelco, Bellafonte, PA) with online detection of the radioactivity, as described previously (16). Protein concentrations were determined with a copper reduction method using bicinchoninic acid, essentially as described by Smith *et al.* (17).

Determination of Intron-Exon Junctions. The amplification of the genomic regions containing the intron-exon boundaries of exon 4, 9, 12, 15, and 21 was performed with the Genomewalk system using nested PCR with the primer sets as specified in Table 1.

PCR Amplification of Coding Exons. DNA was isolated from purified granulocytes by standard procedures. PCR amplification of all of the 23 coding exons and flanking intronic regions was carried out using the primer sets as specified in Table 2. Forward primers of exon 2, 9, 15, and 21 had a 5′-TGTAAAACGACGGCCAGT-3′ extension at their 5′-ends, whereas reverse primers had a 5′-CAGGAAACAGCTATGACC-3′ extension at their 5′-ends. These sequences are complementary to the labeled −21M13 and M13 reversed primers used in the dye-primer sequence reaction. Amplification of all of the exons, except exons 1 and 14, was carried out in 50-μl reaction mixtures containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 10 pmol of each primer, 200 μM each deoxynucleotide triphosphate, and 2 units of Taq polymerase. Amplification of exon 1 was performed in the presence of 0.75 mM $MgCl_2$ and 5% DMSO with a hot start. Amplification of exon 14 was performed in the presence of 2 mM $MgCl_2$. After initial denaturation for 5 min at 95°C, amplification was carried out for 30–35 cycles (1 min at 95°C, 1 min at 50°C–65°C, and 1 min at 72°C). PCR products were separated on 1% agarose gels, visualized with ethidium bromide, and purified using a Qiaquick Gel Extraction kit or used for direct sequencing.

Table 3 Patient characteristics

	Total group (n = 37)	DPD < 70% (n = 22)	DPD > 70% (n = 15)
Age			
Median (yr)	61	59	66
Range	32–79	35–79	32–77
Gender			
Male	15	10	5
Female	22	12	10
Histology			
Colon cancer	23	14	9
Breast cancer	7	4	3
Rectal cancer	6	3	3
Gastric cancer	1	0	1
DPD activity (nmol/mg/h)			
Mean ± SD	6.6 ± 3.7	4.0 ± 1.4	10.5 ± 2.2
Range	0.7–16.4	0.7–6.3	7.6–16.4
Time to onset of toxicity (days)			
Mean ± SD	13.9 ± 12.3	10.0 ± 7.6	19.1 ± 15.3
Range	2–52	2–31	3–52
Duration of toxicity (days)			
Mean ± SD	19.9 ± 12.9	18.2 ± 11.4	20.9 ± 14.7
Range	7–61	9–61	7–61

Sequence Analysis. Sequence analysis of genomic fragments amplified by PCR was carried out on an Applied Biosystems model 377 automated DNA sequencer using the dye-primer or dye-terminator method.

Statistics. Differences in the time of the onset of toxicity and the duration of toxicity between two groups were analyzed with the two sample t test. In case of unequal variances, as indicated by Levene's test for equality of variances, the log-transformed data were used, or the original data were tested with the nonparametric Mann-Whitney test. Comparison of the toxicity between groups was performed with the Mann-Whitney test. Comparison between the DPD activity and the degree of toxicity was performed with a one-way ANOVA. Comparisons of frequencies were carried out by the χ^2 test. The correlation between the activity of DPD and the time of the onset of toxicity was studied by the determination of the Pearson correlation coefficients and linear regression. The level of significance was set *a priori* at $P \leq 0.05$. Analyses were performed with the Statistical Package for the Social Sciences (SPSS Inc., Chicago, IL).

RESULTS

DPD Activity. A large range of DPD activity (0.7–16.4 nmol/mg/h) was observed in PBM cells of patients suffering from unexpected severe toxicity after the administration of 5FU (Fig. 1). On the basis of previous experiences, it has been suggested that patients with a DPD activity <70% of that observed in the normal population might be prone to develop severe 5FU-associated side effects (15). According to this threshold level, in approximately 59% of the cases the DPD activity was <70% of the mean DPD activity (10.0 ± 3.4 nmol/mg/h; n = 22) of healthy volunteers and comparable with that observed in obligate heterozygotes (5.5 ± 2.1 nmol/mg/h; n = 8).

Patient Characteristics and Clinical Presentation. The characteristics of the patients suffering from severe 5FU-associated toxicity are summarized in Table 3. No significant differences were observed with respect to age, gender, or type of histology between patients with a low DPD activity and patients with a normal DPD activity. A significant difference was observed, however, in the time of the onset of toxicity between both groups. In patients with a low DPD activity, the onset of toxicity occurred on average twice as fast as that compared with patients with a normal DPD activity ($P < 0.05$). A weak but significant correlation was observed between the DPD activity and the time of the onset of toxicity (Fig. 2). In contrast, no differences were observed with respect to the duration of the toxicity, which was approximately 20 days.

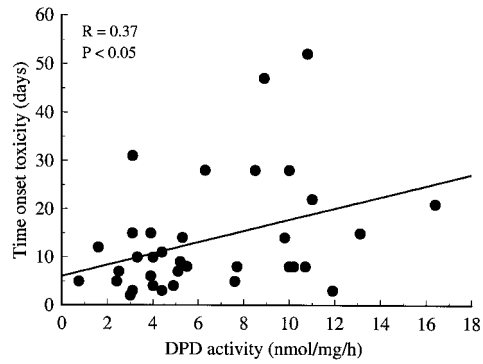

Fig. 2 Correlation between the DPD activity and the time of onset of toxicity. The correlation between the DPD activity in PBM cells and the time of onset of toxicity was studied by determination of the Pearson correlation coefficient and linear regression.

The various types of toxicity encountered in the two groups of patients are shown in Tables 4 and 5. No differences in hematological, gastrointestinal, flu-like symptoms, or other types of toxicities were observed between both groups, with the exception of grade IV neutropenia. It was observed that 55% of

Table 4 Toxicity profile of patients suffering from severe 5FU toxicity[a]

	DPD < 70% (n = 22)				DPD > 70% (n = 15)			
Toxicity	I	II	III	IV	I	II	III	IV
Hematological								
Neutropenia	2	2	1	12	2	3	4	2
Thrombocytopenia	4	2	1	3	1	2	1	1
Others	2	0	2	0	0	1	1	0
Gastrointestinal								
Nausea	4	6	6	0	4	4	1	1
Vomiting	2	5	6	2	3	2	3	0
Diarrhea	4	2	7	6	1	5	2	3
Stomatitis	2	3	7	5	1	1	4	5
Anorexia	2	2	5	2	2	2	2	1
Others	0	0	1	0	1	0	0	0
Flu-like symptoms								
Fever in absence of infection	1	1	1	1	0	0	3	0
Malaise	2	1	1	2	1	4	1	2
Others								
Dermatological	1	2	4	0	1	1	1	2
Neurological	0	1	0	0	1	1	0	1
Alopecia	5	4	2	0	0	1	0	1
Hand-foot syndrome	1	1	0	1	1	0	1	1
Others	0	1	3	1	0	0	2	0
Total[b]	32	33	47	35	19	27	26	20

[a] The figures represent the number of patients suffering from a particular type of toxicity (graded according to the Common Toxicity Criteria).
[b] Total number of toxicity events.

Table 5 Clinical presentation of patients suffering from severe 5FU toxicity[b]

	DPD < 70% (n = 22)					DPD > 70% (n = 15)				
Toxicity	0	I	II	III	IV	0	I	II	III	IV
Hematological	5	2	2	1	12	4	2	3	3	3
Gastrointestinal	0	1	4	9	8	1	0	2	5	7
Flu-like symptoms	15	2	1	2	2	6	1	4	2	2
Others	8	3	4	5	2	5	2	2	3	3

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

the patients with a decreased DPD activity suffered from grade IV neutropenia compared with 13% of the patients with a normal DPD activity (P = 0.01).

Genotype in Partially DPD-deficient Patients. Recently, the genomic structure of the *DPD* gene has been published, including the intron-exon boundaries of the 23 coding exons (18). A close examination of the published sequences showed, however, that a number of primers (exon 4, 9, 15, and 21) were situated inside the exon, thus preventing the detection of putative splice site mutations. Furthermore, a primer of exon 12 proved to be completely identical to a part of the adaptor primer provided by the Genomewalk kit. For these reasons, we have determined the intron-exon boundaries of exon 9, 12, 15, and 21 (Table 6). In addition, the sequences flanking exon 4 were determined to allow the detection of the frameshift mutation 295–298delTCAT. Sequence analysis of the *DPD* gene of multiple patients revealed sequence errors in the previously reported sequences of the intron-exon boundaries of exon 2, 4, 5, 8, 15, 17, 19, and 21. The correct sequences flanking the 23 exons of the *DPD* gene are listed in Table 6. As a result, different primer sets were used to amplify the 23 exons of the *DPD* gene (Table 2).

Analysis of the *DPD* gene of 14 patients with a reduced DPD activity revealed the presence of mutations in 11 of 14 patients (Table 7). Furthermore, five patients had multiple mutations in the coding region of the *DPD* gene. The missense mutation 85T→C was detected in four patients (29%), whereas the splice site mutation IVS14+1G→A was detected in six patients (43%). One patient was homozygous for the 2194G→A mutation, whereas three patients were heterozygous for the 1627A→G mutation. It has been suggested that both the 2194G→A mutation and the 1627A→G mutation are common polymorphisms (11, 13). In addition, two novel mutations 496A→G (M166V) and 2846A→T (D949V) were detected in exon 6 and 22, respectively. The D949V mutation has also been detected in a patient with a complete DPD deficiency.[3] In three patients, no mutations could be detected in the coding sequences of the *DPD* gene.

[3] Unpublished results.

Table 6 Splice junction sequences of the 23 exons of the *DPD* gene

Exon	3′ Acceptor site[a]	5′ Donor site[a]
1		CGAG**gt**acggacttcgcgcc
2	tttatgctgtctttag**AG**TA	CTTT**gt**aagtaccactgata
3	tttaaatatgttgc**ag**AATT	TGAG**gt**aagtctgggtcacc
4	ttattttatttcct**ag**ATGC	CAAG**gt**aaattcagatttaa
5	tgtattaattttgc**ag**AACT	TGAG**gt**atgtatgatataca
6	attgatttccccgt**ag**GTAT	TAAG**gt**aatgcctacattta
7	tctcctttctttc**ag**TACT	AAAG**gt**aaatgaaaaaaaca
8	atattgtttcttat**ag**ATAA	ATAG**gt**gagtagtttactct
9	aaatttgattactt**ag**GTTT	GCAG**gt**ataacatattgtct
10	tcctttcatcattc**ag**GAAT	GGAG**gt**aaaatggaaccatc
11	ttctgttttgttttag**AT**GG	AAAG**gt**acagtgctgggagc
12	tcacttgttttttc**ag**TAAA	ACAG**gt**aggcatttgccatc
13	ggtttgtattttgc**ag**TCAC	TAAG**gt**aagaaaatattatt
14	tttactctttcatc**ag**GACA	CAAC**gt**aagtgtgatttaac
15	gtttttctttttaa**ag**ATTG	TGAG**gt**aatggttactttag
16	cctttcttgtttaa**ag**GATT	GCAG**gt**aaggaccttgacag
17	cgacctatttgaac**ag**GATC	GAAG**gt**aagaacttgacttg
18	gatgtgtcttgcat**ag**GTGG	TCTG**gt**aggtgttgcccact
19	ttctgattttgtgt**ag**GGAC	CCAG**gt**agtcattgtgtttg
20	gctctgtctttttc**ag**GTAT	CAAG**gt**atgtgcttaaatct
21	tgtgttttccttttagAAAC	CAAG**gt**aaaaattatgccaa
22	cagtggctatttttagGATG	CCAG**gt**aagaatcctgctgg
23	ctatttctgtttgc**ag**GCTA	

[a] Exon and intron sequences are denoted by uppercase and lowercase letters, respectively, and the nucleotide consensus sequence of the intron adjoining the splice junctions are in bold.

DISCUSSION

In this study, we have evaluated the role of a partial DPD deficiency in the etiology of unexpected severe 5FU toxicity encountered in 37 cancer patients. DPD is generally considered to be the rate-limiting step in the catabolism of the pyrimidine bases uracil, thymine, and the thymine analogue 5FU. Under normal conditions, a low DPD activity is still sufficient to maintain uracil homeostasis because obligate heterozygotes do not excrete elevated levels of pyrimidine bases (20). After the loading of these patients with uracil or thymine, however, the accumulation of pyrimidine bases in plasma and urine increased compared with normal individuals, indicating a decreased capacity in heterozygotes to degrade the pyrimidine bases (20). We have recently demonstrated that in a patient heterozygous for a mutant *DPD* allele, the AUC of 5FU in plasma was strongly increased compared with controls.[3]

To date, a number of patients with a partial DPD deficiency have been reported to suffer from severe toxicity after the administration of 5FU. On the basis of these experiences, a threshold limit for the DPD activity (70% of the mean DPD activity of a control population) has been proposed for patients at risk (15). According to this threshold level, 59% of our patient group suffered from a partial DPD deficiency with a mean DPD activity comparable with that of obligate heterozygotes. Thus, the decreased capacity to degrade 5FU, reflected by a decreased activity of DPD, might be directly responsible for the observed toxicity in these patients. Recently, Milano *et al.* (15) reported that in their group of patients, only 36% suffered from a partial DPD deficiency. In contrast, our results demonstrate that in our group of patients, a partial DPD deficiency is the major determinant of 5FU-associated toxicity. Nevertheless, other factors, *e.g.*, an increased expression of enzymes of the anabolic pathway, might provide a complementary mechanism for the observed increased sensitivity of patients to 5FU, especially in those patients with a normal DPD activity.

To date, conflicting results exist as to whether the activity of DPD might be influenced by gender (7, 15). It has been suggested that women might be particularly prone to the development of 5FU-associated toxicity because studies show that the DPD activity as well as the clearance of 5FU is, on average, 15% lower in women than in men (7, 21). In addition, the vast majority (79%) of the patients suffering from a partial DPD deficiency and severe adverse effects of 5FU are women (15). In contrast, we did not observe a difference in gender between the patients with a low DPD activity and patients with a normal DPD activity. Thus, the prevalence of women with a partial DPD deficiency in the study reported by Milano *et al.* (15) remains enigmatic.

With respect to toxicity, no differences in hematological toxicity, gastrointestinal toxicity, flu-like symptoms, or other types of toxicities were observed between patients with a low DPD activity and patients with a normal DPD activity, with the exception of grade IV neutropenia. A significantly higher percentage (55%) of the patients with a decreased DPD activity suffered from grade IV neutropenia, compared with patients with a normal DPD activity (13%). A recent analysis of the toxicities encountered in cancer patients treated with 5FU showed that hematological toxicity, mainly neutropenia, is observed more frequently in patients treated with 5FU bolus than in patients receiving 5FU via continuous infusion (22). Furthermore, no differences in risks of severe diarrhea, nausea, vomiting, and mucositis were observed between the two types of 5FU administration (22). Thus, the higher incidence of grade IV granulopenia in patients with a low DPD activity might be related to the increased plasma levels of 5FU, mimicking the short peak plasma concentrations of 5FU that result from conventional i.v. bolus administration of 5FU. Although neurotoxic syndromes were frequently encountered in a group of patients with decreased DPD activity (15), they appeared to be very rare in our patient population. Our findings, however, are in line with the fact that neurotoxicity is rarely reported as one of the adverse side effects of 5FU.

A conspicuous finding was that the onset of toxicity occurred approximately twice as fast in patients with a partial DPD deficiency compared with patients with a normal DPD activity. A close relationship has been demonstrated to exist between the steady-state 5FU plasma concentration as well as the AUC of 5FU in plasma and the risk of leukopenia and mucositis (3). A sustained high level of 5FU attributable to a decreased capacity to degrade the drug might have accelerated the onset of the clinical symptoms in patients with a low DPD activity. Recently, Etienne *et al.* (23) have shown that not only the DPD activity but also other factors, such as age, high serum alkaline phosphatase, and elapsed time during infusion, are independent covariables that influence the clearance of 5FU. Thus, it is conceivable that these factors also play a role in the time of onset of toxicity. The late onset of the clinical symptomatology might also allow sufficient time to apply dose adaptation schedules. Indeed, controlling the AUC by adjusting the dose of 5FU in the middle of a 5-day infusion resulted in a significant decrease in toxicity (24).

Table 7 DPD activity and DPD genotype in patients with a partial DPD deficiency[a]

Patient no. [sex, age (yrs)]	Primary cancer	DPD activity (nmol/mg/h)	Genotype[b]	Effect[c]	Location[d]
1 (M, 63)	Colon	0.74	85T→C	C29R	EX2
			496A→G	M166V	EX6
2 (F, 49)	Breast	1.6	*1627A→G*	*I543V*	*EX13*
3 (F, 66)	Breast	3.1	IVS14+1G→A	Del (exon 14)	IVS14
4 (M, 51)	Rectal	3.1	85T→C	C29R	EX2
			496A→G	M166V	EX6
			2846A→T	D949V	EX22
5 (F, 57)	Colon	3.9	IVS14+1G→A	Del (exon 14)	IVS14
6 (F, 40)	Colon	4.0	IVS14+1G→A	Del (exon 14)	IVS14
			85T→C	C29R	EX2
7 (M, 79)	Colon	4.4	IVS14+1G→A	Del (exon 14)	IVS14
8 (F, 47)	Colon	4.9	ND[e]		
9 (M, 54)	Colon	5.1	IVS14+1G→A	Del (exon 14)	IVS14
10 (M, 66)	Colon	5.2	ND		
11 (M, 66)	Colon	5.3	85T→C	C29R	EX2
			1627A→G	*1543V*	*EX13*
12 (F, 60)	Colon	5.5	IVS14+1G→A	Del (exon 14)	IVS14
			1627A→G	*1543V*	*EX13*
13 (F, 63)	Colon	5.9	ND		
14 (M, 53)	Rectal	6.3	*2194G→A*	*V732I*	*EX18*
			2194G→A		

[a] Known polymorphisms are written in italic.
[b] Nomenclature according to Antonarakis (19).
[c] Effect of the mutation on DPD protein or mRNA.
[d] According to Wei *et al.* (18).
[e] ND, not detected.

The human *DPD* gene consists of 23 exons and is at least 950 kb in length, with 3 kb of coding sequence and an average intron size of about 43 kb (18). To date, 14 different mutations have been identified in the *DPD* gene, and the vast majority of these mutations have been detected in patients with a complete DPD deficiency, which was accompanied by a wide variety in clinical presentation (12). To understand the genetic basis for the partial DPD deficiency of 14 cancer patients with a reduced DPD activity, we have analyzed the coding exons of the *DPD* gene for the presence of mutations. In this group of patients, six different mutations were identified including: (*a*) one splice site mutation (IVS14+1G→A); (*b*) one missense mutation (85T→C); (*c*) two polymorphisms (1627A→G and 2194G→A); and (*d*) two novel mutations (496A→G and 2846A→T).

The missense mutation 85T→C has been identified previously in a homozygous state in two patients with a complete deficiency of DPD, and a functional analysis of this mutation in *Escherichia coli* demonstrated that the C29R mutation resulted in a mutant DPD protein without significant residual enzyme activity (25). Recently, however, it has been suggested that the C29R mutation might be a common polymorphism because a high frequency has been noted for this mutation in a population of cancer patients, including homozygosity for this mutation in two individuals with almost normal DPD activity (11). The apparent discrepancy between our results and those reported by Collie-Duguid *et al.* (11) remains enigmatic.

In three patients, no mutations could be detected in the coding sequences of the *DPD* gene. We cannot exclude, however, the possibility that these patients might be heterozygous for a mutation in the promoter region of the *DPD* gene. Recently, a polymorphism that disrupts a putative γ-IFN response element was identified in a cancer patient with reduced DPD activity (26).

Analysis of the prevalence of the various mutations among cancer patients with a partial DPD deficiency showed that the G→A point mutation in the invariant splice donor site is the most common one (43%). This observation is in line with the fact that the IVS14+1G→A mutation is also the most predominant mutation detected in patients with a complete DPD deficiency (12). Surprisingly, the screening of 23 patients with a reduced DPD activity identified only 1 patient heterozygous for the common splice mutation (11). Our results demonstrate that at least 57% (8 of 14) of the patients with a reduced DPD activity have a molecular basis for their deficient phenotype.

In conclusion, a partial DPD deficiency appears to play an important role in the etiology of 5FU-associated toxicity. Considering the common use of 5FU in the treatment of cancer patients, the severe 5FU-related toxicities in patients with a low activity of DPD, and the high frequency of the IVS14+1G→A mutation in DPD-deficient patients, it would be preferable for the analysis of DPD activity in PBM cells or screening for the IVS14+1G→A mutation to be routinely carried out before the start of treatment with 5FU.

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Clinical Implications of Dihydropyrimidine Dehydrogenase (DPD) Deficiency in Patients with Severe 5-Fluorouracil-associated Toxicity: Identification of New Mutations in the *DPD* Gene

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